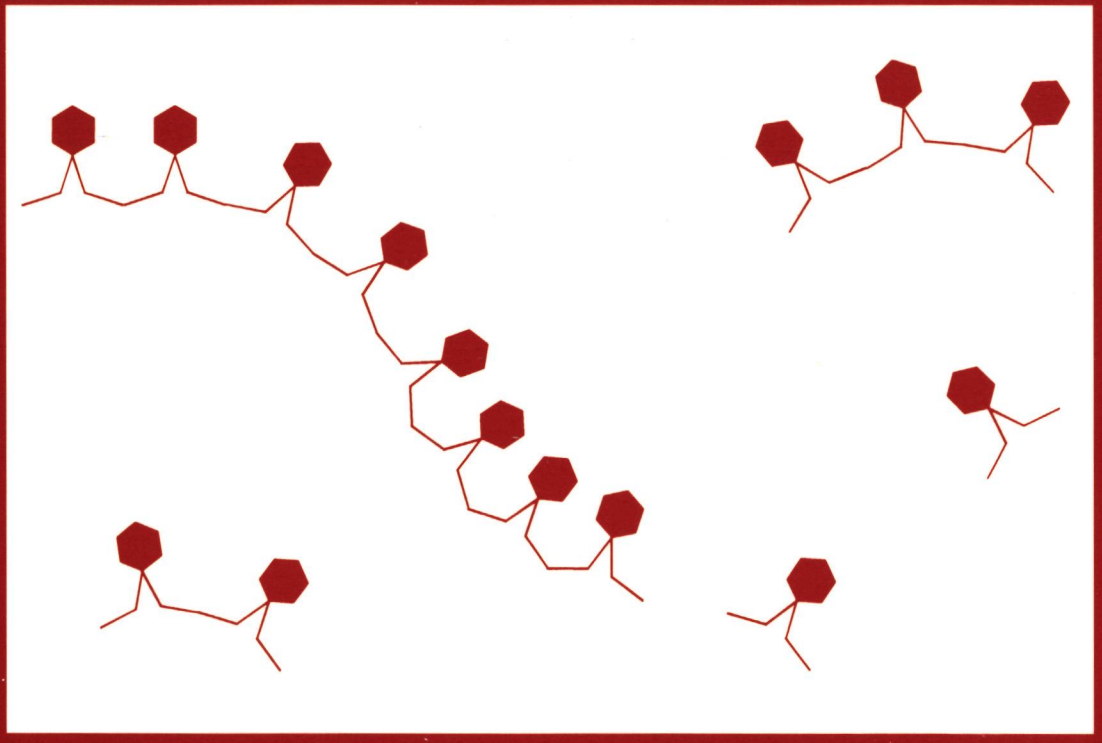
Acyclic Nucleotide Analogues: Synthesis and Oligomerization Studies of Disubstituted Pyrimidines



Michiel J. van Vliet

Acyclic Nucleotide Analogues: Synthesis and Oligomerization Studies of Disubstituted Pyrimidines

Acyclic Nucleotide Analogues: Synthesis and Oligomerization Studies of Disubstituted Pyrimidines

een wetenschappelijke proeve
op het gebied van de Natuurwetenschappen

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Michiel Johan van Vliet

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Promotor: Prof. Dr. A.W. Schwartz

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The pleasure of discovery in science derives not only from the satisfaction of new explanations, but also, if not more so, in fresh (and often more difficult) puzzles that the novel solutions generate.

(Stephen Jay Gould)

Voor Mignon en mijn ouders

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Contents

Abbreviations

A,	adenosine
Ad,	adenine
AppA,	diadenosine-5',5'-pyrophosphate
C,	cytidine
Cy,	cytosine
$\underline{C}$,	1-[(1,3-dihydroxy-2-propoxy)methyl]cytosine
DMF,	dimethylformamide
DMSO,	dimethylsulfoxide
EDAC,	1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide hydrochloride
G,	guanosine
Gu,	guanine
$\underline{G}$,	9-[(1,3-dihydroxy-2-propoxy)methyl]guanine
HPLC,	high performance liquid chromatography
Im,	imidazole
Imp$\underline{G}$pIm,	the bisphosphoimidazolide of $\underline{G}$
KEDTA,	dipotassium salt of ethylenediaminetetraacetic acid
2,6-lutidine,	2,6-dimethylpyridine
MeIm,	2-methylimidazole
NaAc,	sodium acetate
NMR,	nuclear magnetic resonance
p$\underline{C}$p,	the bisphosphate of $\underline{C}$
poly(C),	polycytidylic acid
poly(U),	polyuridylic acid
pyr,	pyridine
TEA,	triethylamine
TEAB,	triethylammonium bicarbonate
TMS,	tetramethylsilane
Tris,	tris(hydroxymethyl)aminomethane
U,	uridine
ODU,	optical density unit

Introduction

The RNA World.

It is widely believed that the first living cells on earth were preceded by a set of informational macromolecules. These molecules had to be self-replicating, as no individual copies would otherwise be left when they were chemically degraded. They also had to express their genetic information as a behavioral phenotype, so that a useful variation in the genotype could be assessed by natural selection. In this way more functional variants were favored over less functional ones. The most attractive candidates for the role of informational macromolecules are those compounds that have inherent template properties, such as the nucleic acids: DNA and RNA. Therefore in the late sixties the idea was proposed that the first self-replicating system consisted of a set of RNA molecules (Woese, 1967; Crick, 1968; Orgel, 1968). A number of arguments for the hypothesis that life was based on RNA and not DNA have been given. When *E. coli* bacteria are infected with the RNA virus Qβ, a single enzyme is responsible for the replication of the viral genome in contrast to the multiple enzyme systems needed to carry out DNA replication (Eigen et al., 1981). Furthermore, RNA plays a central role in biological systems, especially in important processes such as replication and translation of genetic information. For example, RNA primes DNA synthesis during DNA replication. Genetic information is transcribed from DNA into messenger RNA. Messenger RNA carries the genetic information from DNA to the translation apparatus (ribosomes), which consist of RNA-protein structures. In the ribosomes transfer-RNA molecules position the amino acids in accordance with the codon sequence of messenger RNA.

However, reconstructing a replicating system from RNA appeared to be a "chicken and egg" problem. Only nucleic acids seemed capable of storing and replicating genetic information, and only proteins were known to function as catalysts. No example of RNA involved as catalyst in any reaction was known. The discovery of catalytic RNA by Altman and Cech (Kruger et al., 1982; Guerrier-Takada et al., 1983) has overcome this problem and has made the idea of self-replicating RNA increasingly popular. RNA is the only set of molecules that is known to function as both phenotype and genotype and, therefore, replication of RNA enables Darwinian evolution to occur at the molecular level. This principle was demonstrated by Eigen and coworkers in replication experiments of RNA with the enzyme Qβ replicase in cell-free systems (Eigen et al., 1981). The discovery of catalytic RNA molecules led to the idea of an "RNA world" (Gilbert, 1986). RNA world hypotheses are all based on three assumptions (Joyce and Orgel, 1993): (1) At some stage in evolution replication of RNA was responsible for the translation of genetic information; (2) Recognition of the molecules in the replication process was by Watson-Crick base pairing; (3) Genetically encoded proteins were not involved as catalysts. It should be emphasized it is unclear if life started with an RNA-world, however, growing lines of evidence suggest that an RNA-world existed as a

precursor of our DNA/protein world. One striking example is the demonstration that the peptide bond-forming step in protein synthesis is catalyzed by largely protein-free ribosomal RNA (Noller et al., 1992).

The synthesis of oligonucleotides.

The RNA world hypothesis requires that RNA-like molecules were formed spontaneously under prebiotic conditions. In order to simulate the synthesis of oligonucleotides on the prebiotic earth, the nonenzymatic oligomerization of activated β-D-nucleoside-5'-phosphates has been studied. This reaction was performed using contemporary nucleotides as starting material, although the abiotic synthesis of these compounds is still doubtful and will be discussed later on. In the enzymatic synthesis of polynucleotides, the activated monomers are nucleoside-5'-triphosphates, which react inefficiently in the absence of an enzyme. Prebiotically plausible molecules, such as cyanamide and cyanoacetylene, are able to activate nucleotides. However, condensation reactions of nucleotides with those molecules are also inefficient (Lohrman and Orgel, 1973). Imidazole has been used successfully as an activating agent. Although imidazole is only marginally plausible as a prebiotic molecule, nucleotides are easily converted to nucleoside-5'-phosphoimidazolides (Figure 1), which react efficiently in aqueous solution.

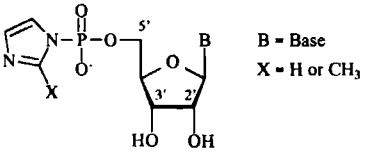


Figure 1. Structure of an activated nucleotide as used in nonenzymatic oligomerization reactions. R=H, nucleoside 5'-phosphoimidazolide; X=CH_3, nucleoside 5'-phospho-2-methylimidazolide.

The reactivities of both the 5'-phosphate and 2'-OH groups are higher than that of the 3'-OH group and, as a result, reactions of activated nucleotides yield products containing one 5',5'-pyrophosphate and a preponderance of 2',5'-phosphodiester bonds (Figure 2). Several metal ions catalyze the formation of oligonucleotides from nucleoside 5'-phosphoimidazolides and increase the extent of 3',5'-phosphodiester bonds. However, the products still contain a substantial proportion of 2',5'-phosphodiester bonds (Sawai, 1976; Sleeper and Orgel, 1979). For example, Sawai showed that short oligomers of the four natural nucleotides are produced with Pb^{2+} as catalyst, although 2',5'-bonds predominate (Sawai, 1988).

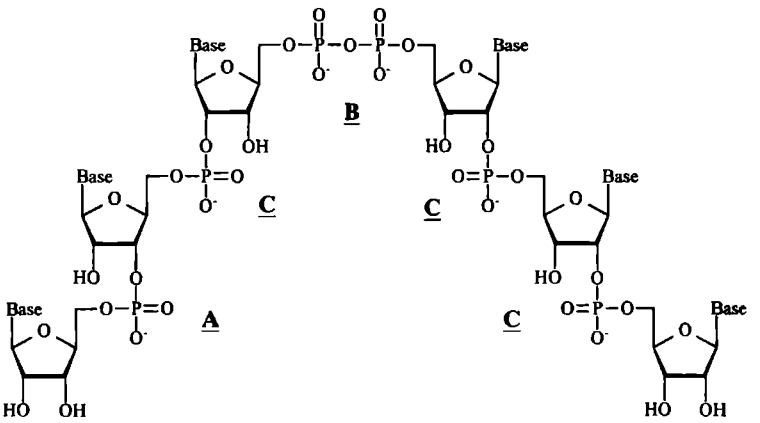


Figure 2. Example of an oligonucleotide product resulting from condensation of activated mononucleotides. The hexanucleotide contains one 3',5'-phosphodiester (A), one 5',5'-pyrophosphate (B) and three 2',5'-phosphodiester (C) linkages.

In the oligomerization reactions described above, relatively high concentrations (0.05 M) of monomers were used. Since it is unlikely that high concentrations of the nucleotide starting materials were present on the prebiotic earth, some process was probably responsible for concentration of these molecules from dilute aqueous solution. In 1949 Bernal proposed that clay minerals may have served to concentrate and orient small molecules for reaction and protect adsorbed molecules from degradation. Some of these aspects of clay minerals have been investigated extensively by Ferris and coworkers. They used montmorillonite clays to adsorb nucleotides and catalyze the synthesis of oligonucleotides. Although the formation of phosphodiester bonds was increased, a substantial amount of products containing pyrophosphate and 2',5'-bonds was still formed (Ferris et al., 1989; Ferris and Ertem, 1992a; Kawamura and Ferris, 1994). In more recent work, conditions have been found, including the use of a special activating group, which lead to the formation of 3',5'-linked oligonucleotides with a regioselectivity averaging 88%. No pyrophosphate-capped products were detected (Prabahar et al., 1994). In the presence of montmorillonite, activated mononucleotides containing G or A can be oligomerized in good yield. When nucleotides based on U or C are reacted, however, only small amounts of oligomers up to approximately trimer have been produced (Ferris and Ertem, 1992b). In conclusion, oligomers containing purine residues can be formed, but with moderate stereospecificity. Oligomerization of activated nucleotides containing pyrimidines is very inefficient and forms an obstacle in the abiotic synthesis of RNA molecules.

Template-directed synthesis of oligonucleotides

Whenever oligonucleotides became available the next stage in the chemical evolution of an RNA world would have been the replication of some of these molecules. In order to mimic this replication Orgel developed the template-directed synthesis of oligonucleotides. In this reaction activated mononucleotides are oriented along a complementary oligonucleotide, favoring the formation of phosphodiester bonds (Figure 3). The complex formation is based

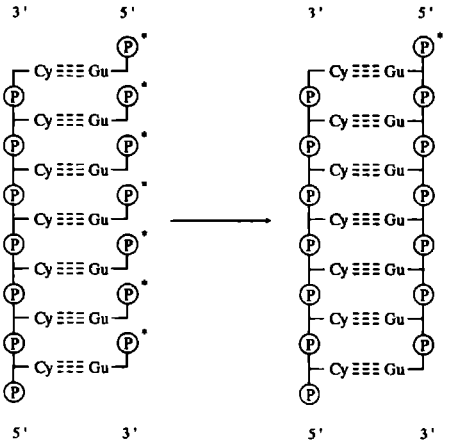


Figure 3. Schematic illustration of the template-directed oligomerization. In this example a poly(C) template forms a double helical complex with activated derivatives of guanosine-5'-phosphate. The orientation of the phosphate groups facilitate the formation of phosphodiester bonds (form Orgel, 1986).

on Watson-Crick base pairing - accompanied under some conditions by Hoogsteen base pairing - and is stabilized by stacking interactions between the base units of each strand. In early work, the products always contained a mixture of 2',5' and 3',5'-phosphodiester bonds (Sleeper and Orgel, 1979; Inoue and Orgel, 1981). More recently, the oligomerization of guanine 5'-phospho(2-methyl)imidazolides directed by poly(C) has been shown to be highly stereospecific and the fidelity of the reaction (i.e., the probability that a complementary base rather than a noncomplementary base will be incorporated opposite a given base in the template) is excellent. Thus, if poly(C) is incubated with an equimolar mixture of all four activated ribonucleotides (A, G, C and U), less than 1% of A, C or U is incorporated in the product oligomer (Inoue and Orgel, 1982). Random copolymers containing as little as 60% of C-residues are still effective as templates for the synthesis of products containing G and the complements of the other bases present in the template (Inoue and Orgel, 1983). It has also been demonstrated that some specific oligonucleotide sequences can be copied faithfully

(Inoue et al., 1984; Acevedo and Orgel, 1987; Wu and Orgel, 1992a). These include sequences containing stretches of C-residues, with occasional A, U and G-residues isolated from each other by at least three C-residues. It is clear, however, that such template-directed oligomerization could not result in a replicating system for two reasons. Firstly, poly(C,G) templates which do not contain an excess of C-residues form stable self-complementary structures that prevent them acting as templates (Joyce and Orgel, 1986). Types of self-complementary structure which are important here include an intermolecular complex involving a tetrahelix formed by sequences of consecutive G-residues as well as an intramolecular complex in which the molecule folds back on itself to form complementary Watson-Crick double-helical segments. Some progress has been made by the discovery (using hairpin loop primers) that short runs of G can be copied into runs of C-residues as long as the formation of self-complementary structures of G-residues can be avoided (Wu and Orgel, 1992b; Rembold and Orgel, 1994). Secondly, a poly(A,U) template with a high content of A-residues will not direct the synthesis of a complementary oligonucleotide, because stacking of the U-residues is too low to form a stable complex with the template (Saenger, 1984).

In summary, synthesis of complementary oligonucleotides can be achieved for some templates containing an excess of C-residues. Replication is more difficult since a pair of complementary sequences cannot be found each of which facilitates the synthesis of the other using activated nucleoside 5'-phosphates as substrates.

Replicating systems.

Several model replicating systems have been described, although their relevance for the origin of life is doubtful. The first successful reaction was demonstated by von Kiedrowski (1986). A self-complementary hexanucleotide derivative was shown to catalyze the formation of an identical hexanucleotide derivative from two trinucleotide residues. Self-association of the hexanucleotide derivatives into double helices was recognized as a problem. To overcome this difficulty it will almost certainly be necessary to cycle the temperature of the reaction mixture or some other variable that controls the stability of double-helical complexes relative to single strands.

Rebek and coworkers have constructed a totally artificial organic molecule, that replicates in organic solution (Tjivikua et al., 1990). This self-replicating compound contains an adenine residue which is attached to a receptor for adenine (Figure 4). Two subunits containing an activated carboxylic acid and a primary amine, respectively, are complexed by the template via H-bonds and stacking interactions. In this complex the orientation of the subunits favors the reaction of the activated carboxylic acid with the primary amine to

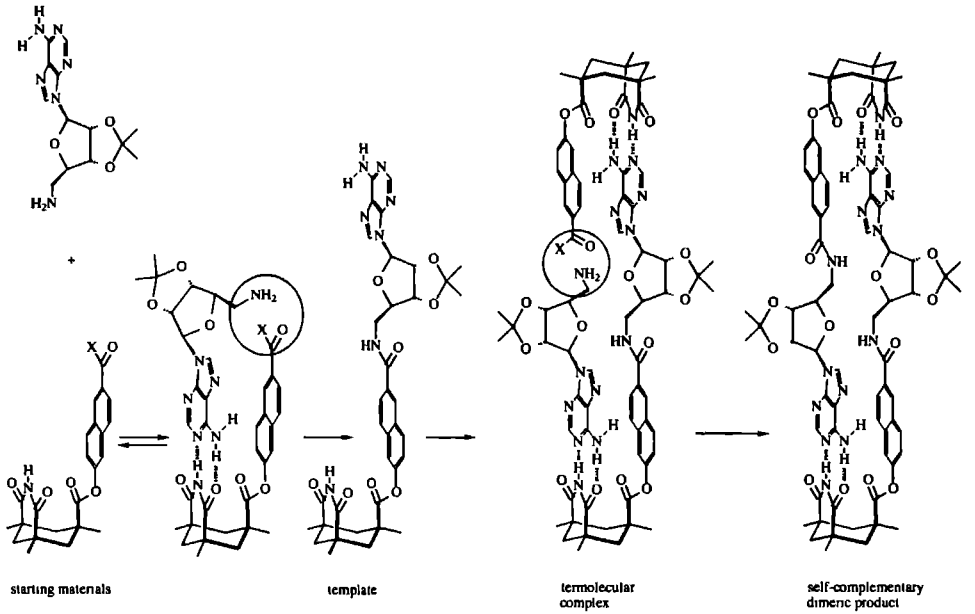


Figure 4. Autocatalysis in a self-replicating system designed by Rebek (1990).

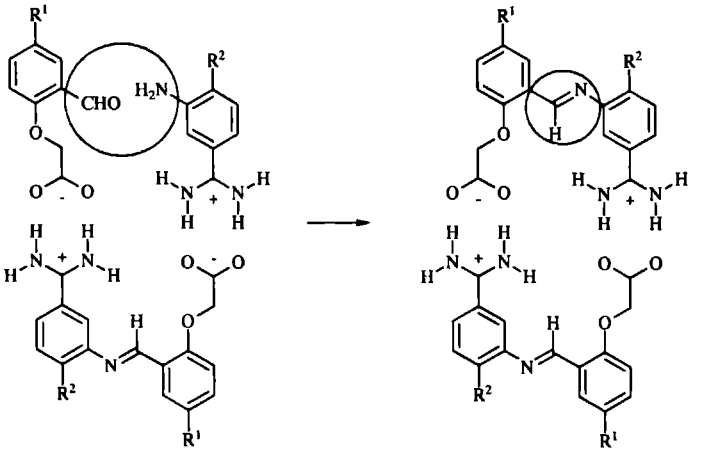


Figure 5. Self-replication based on the formation of a carboxylate-amidinium salt bridge as used by Terfort and von Kiedrowski (1992). For example: $R^1 = NO_2$; $R^2 = tBu$.

generate a molecule identical with the template[1]. Self-association of these molecules is also a limiting factor for their autocatalytic synthesis. A reaction in which this problem has been solved partially was developed by Terfort and von Kiedrowski (1992). They demonstrated that some anils (aromatic substituted imines as depicted in Figure 5) can be synthesized autocatalytically by derivatives of 2-formylphenoxyacetic acid and 3-aminobenzamidine in dimethylsulfoxide. It appears that in some special cases the ternary complex is more stable than the binary complex. Presumably the synthesis of the linkage generates a template mole-

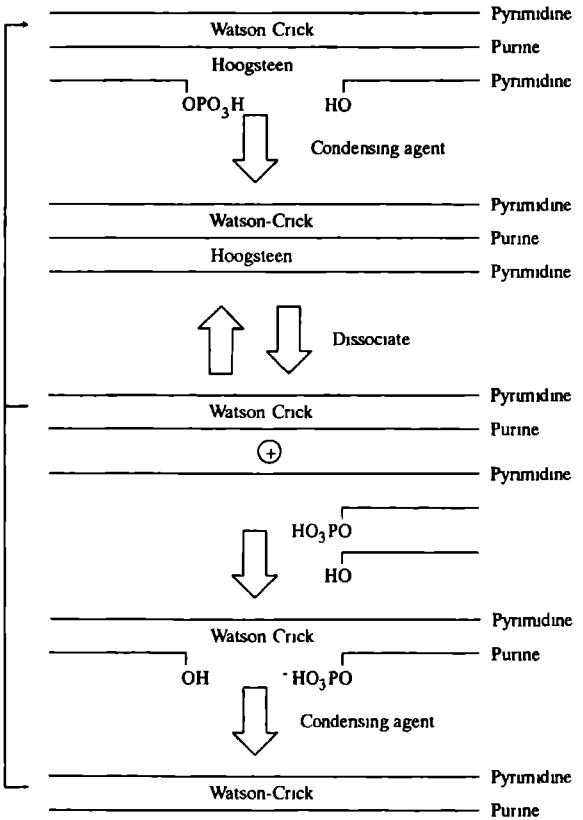


Figure 6. Schematic representation of chemical replication of palindromic DNA via triple helices as demonstrated by Li and Nicolaou (1994) (from Ferris, 1994).

[1]Recently the system of Rebek has been reinvestigated by Menger and coworkers (Menger et al., 1994, Menger et al , 1995). Their experiments showed that a termolecular complex is not a necessary intermediate prior to the transition state and therefore, they have proposed an alternative mechanism involving amide catalysis. The new mechanism accommodates a trivial autocatalysis, but is not "self-replicative" in the usual sence of the word Discussions about the validity of both mechanisms are still going on (Menger et al., 1994; Conn et al., 1994, Menger et al., 1995)

cule that forms less stable salt bridges with itself than with its precursor. Thus a new round of replication is possible, since the problem of self-association is of minor importance.

A very interesting example was reported recently by Li and Nicolaou (1994) involving the self-replication of palindromic (symmetrical) duplex DNA-like oligonucleotides (Figure 6). The duplex consists of a pyrimidine and a purine strand, each of which is 24 units long, bound by Watson-Crick base pairing. This duplex DNA serves as a template for the ligation of two dodecameric pyrimidine fragments via the formation of a triple helix. The newly formed strand is identical with the pyrimidine strand of the original duplex. In the triple helix the newly formed strand is bound to the original DNA by weak Hoogsteen base pairs. Raising the pH releases the newly generated strand from the double helical template, thus allowing it to serve as a template for duplex formation in the presence of two complementary purine dodecameric fragments. After ligation a new strand is produced which is identical to the purine strand of the original DNA. Li and Nicolaou have demonstrated a replicating system that operates by complementarity, as DNA replication in living systems, rather than self-complementarity as in the examples above. Another example of replication utilizing complementary strands was demonstrated by Sievers and von Kiedrowski (1994). They developed a replicating system based on cross-catalytic template-directed synthesis of hexadeoxynucleotide analogues from aminotrideoxynucleotides (Figure 7).

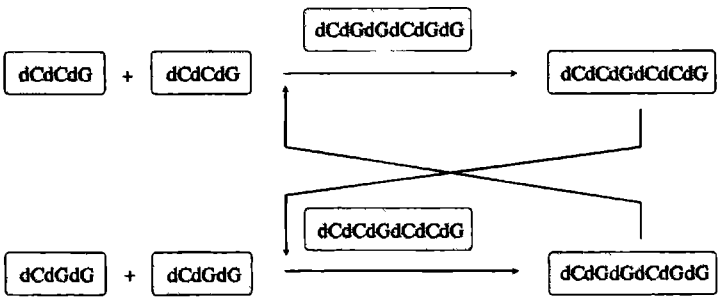


Figure 7. Cross-complementary replication of hexameric DNA oligomers as used by Sievers and von Kiedrowski (1994) (from Ferris, 1994).

In some of the approaches described above it was possible to replicate an oligomer that contains as many purines as pyrimidines. However, those results do not solve all of the problems of replicating RNA in the proposed RNA world. In the work of von Kiedrowski, for example, protecting groups were used to permit the condensation reaction to proceed in only one direction. The replication studied by Li and Nicolaou was performed with symmetrical

DNA to achieve a product strand identical to the starting pyrimidine oligomer. Otherwise a second cycle and appropriate substrate fragments would be required to produce an identical copy of the original template. Nevertheless, the results imply that at least a limited informational replication is possible. The most important problem remaining is perhaps the abiotic synthesis of the mononucleotides and their oligomerization to the starting oligonucleotide sequences.

Prebiotic synthesis of ribonucleotides.

One of the major problems in chemical evolution is that of understanding the prebiotic synthesis of the ribonucleotides. It is implausible that substantial quantities of relatively pure β-D-ribonucleotides could have accumulated on the prebiotic earth. This problem has been defined by Joyce and Orgel as "The prebiotic chemist's nightmare" (1993). The prebiotic synthesis of some of the nucleoside bases seems less problematic. Adenine is produced under mild conditions by oligomerization of HCN, and uracil and cytosine are formed by reactions of cyanoacetylene with urea or guanidine (Ferris et al., 1974; Miller et al., 1987). All these starting materials are prebiotically quite reasonable. A convincingly prebiotic synthesis of guanine has never been reported. However, this purine has been detected in carbonaceous chondrites (a type of meteorite) (Stoks and Schwartz, 1981).

The formation of ribose is ascribed to the formose reaction. This is a base-catalyzed polymerization reaction of formaldehyde in which a large variety of racemic carbohydrates are formed, including a small proportion of ribose (Reid and Orgel, 1967). It seems implausible that D-ribose could have been separated from such a pool of sugars (Shapiro, 1986; Shapiro, 1988). Another approach to the formation of ribose was taken by Eschenmoser and his colleagues. They have shown that the base-catalyzed aldomerization of glycolaldehyde phosphate in the presence of a half-equivalent of formaldehyde yields a relatively simple mixture of tetrose- and pentose-diphosphates and hexose-triphosphates, of which ribose 2,4-diphosphate is the major component (Müller et al., 1990). More recently, they have refined the reaction of glycolaldehyde phosphate with glyceraldehyde phosphate at low concentration and in the presence of layered hydroxides such as hydrotalcite (magnesium aluminium hydroxide) (Pitsch, 1993). This route to ribose 2,4-diphosphate proceeds under mild conditions probably because the substrates are concentrated in the interlayer of the mineral and the positive charge on the metal hydroxide layers favors ionization of the glycolaldehyde phosphate. The prebiotic formation of glycolaldehyde phosphate is still unclear. A potentially prebiotic pathway from aziridine 2-carbonitrile to glycolaldehyde phosphate has been suggested. In addition Pitsch (1993) has demonstrated that glycolaldehyde phosphate can be synthesized from oxirane carbonitrile and inorganic phosphate in the presence of formaldehyde under weakly alkaline

conditions. However, it is unclear if oxirane carbonitrile can be synthesized prebiotically (Pitsch et al., 1994).

The condensation of purine bases with ribose has been achieved by heating in the presence of inorganic salts (Fuller et al., 1972). Low yields of α- and β-nucleosides are obtained in this way, whereas in the case of pyrimidine bases no nucleosides have been detected.

Was RNA preceded by another system?

It has become clear that the prebiotic synthesis of β-ribosides can not be achieved by presently known pathways in reasonable yields and without the formation of a large amount of side products. The complex mixture of products is a serious problem, since there is no plausible mechanism for the separation of one isomer from a pool of closely related isomers. Experiments performed by Joyce showed that the poly(C)-directed polymerization of racemic guanine 5'-phospho(2-methyl)imidazolide is very inefficient, since incorporation of the L-isomer inhibits the reaction (Joyce et al., 1984). It has been suggested that Watson-Crick base pairing between the L-isomer and the template is only possible when the L-isomer adopts the *syn*-conformation, resulting in an unfavorable orientation of the 3'-OH group (enantiomeric cross-inhibition). Because of the problems associated with the prebiotic synthesis of the β-nucleotides and the above mentioned enantiomeric cross-inhibition in template-directed reactions, it was proposed that RNA was preceded by a more primitive polymer. This precursor may have been constructed of flexible, acyclic, probably prochiral nucleotide analogues that were synthesized readily on the primitive earth (Joyce et al., 1987). Examples of such analogues are depicted in Figure 8. The use of acyclic prochiral nucleotide analogues eliminates the problem of stereochemical inhibition, but it raises a completely equivalent problem. When a monomer is incorporated in the growing polymer, the central C-atom of each nucleotide

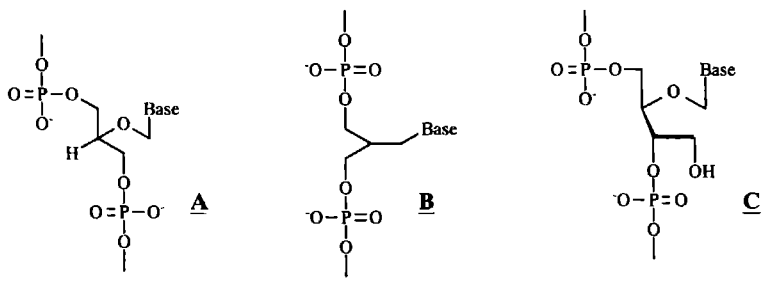


Figure 8. Structures of three acyclic nucleotide analogues. Compounds A and B are prochiral. A, glycerol-derived acyclonucleoside; B, acrolein-derived nucleoside analogue; C, erythritol-derived nucleoside.

analogue becomes chiral. This results in two possible enantiomers of each monomer in a polymer chain. Model building suggests that the conformations of the two enantiomers can be interchanged without significant distortion of the chain. A polymer of acyclic derivatives will contain atactic regions, e.g. regions were both enantiomers are present. Polymers with atactic regions may be able to function as templates in oligomerization reactions of complementary analogues, if they are sufficiently flexible. Template-directed oligomerization experiments were performed to test this hypothesis and the results are described later on in this chapter.

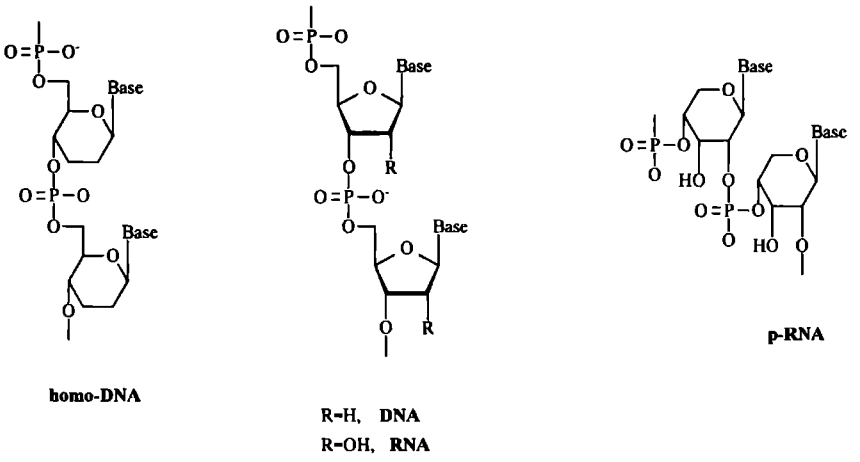


Figure 9. Constitution and configuration of homo-DNA in comparison to DNA, and p-RNA in comparison to RNA.

Other investigators have also begun to search for genetic systems based on modified RNA molecules. When Eschenmoser and colleagues studied the synthesis of sugar phosphates from glycolaldehyde phosphate in the absence of formaldehyde, they observed the selective formation of *rac*-hexose 2,4,6-triphosphates. Because of this and other observations Eschenmoser asked the question: "Why did nature choose pentoses and not hexoses as the sugar building blocks of nucleic acids?" (Eschenmoser and Dobler, 1992). In a model study he and his coworkers investigated the pairing properties and structure of β-D-2',3'-dideoxyglucopyranosyl (6'→4') oligonucleotides ('homo-DNA') (Figure 9). They found that the pairing properties between the bases of this system are uniformly stronger than the pairing in the corresponding DNA oligonucleotides (Hunziker et al., 1993). Double strands of homo-DNA form a quasi-linear structure instead of the helical structure that is formed by DNA. Conformational analysis of both structures revealed that the helicity of DNA duplexes is primarily a result of the five-memberedness of the sugar ring. This conformational analysis also

predicted the existence of new pairing systems. Most interestingly it predicted the existence of a ribopyranosyl isomer of RNA ('p-RNA') containing the phosphodiester linkage between the positions C(4') and C(2') of neighboring ribopyranosyl units (Figure 9). Experiments with β-D-ribopyranosyl (4'→2') oligonucleotides derived from adenine and uracil showed that adenine-uracil pairing in p-RNA duplexes is stronger than in the corresponding RNA duplexes (Pitsch et al., 1993). Significantly, an octamer of p-RNA, containing only adenine bases, did not show self-pairing. If conditions can be found for the simple synthesis of ribonucleotides containing a six-membered sugar ring, p-RNA is a promising candidate for an earlier genetic system.

Template-directed reactions with modified nucleotides.

Essential for an informational replicating system is that the transfer of information is based on interaction (recognition) between two chains, which have to consist of at least two different monomeric units (Orgel, 1992). Furthermore, it is important that the template chain directs the monomeric units into a favorable orientation for reaction. It appears to be difficult to design a novel informational replicating system, since model studies almost always use variations on RNA. Because the informational transfer properties of RNA are largely due to the properties of the bases themselves, most template-directed reactions of modified analogues have been performed with alterations in the newly formed backbone structure, while the bases remain unaltered. In a few studies the base was replaced by another heterocyclic residue, but this was always a closely related purine. An example that showed unexpectedly small catalysis in the template-directed oligomerization of modified heterocyclic bases was demonstrated by Webb and Orgel (1982). They studied the reaction of 2-aminoadenosine-5'-phosphoimidazolides in the presence of poly(U). 2-Aminoadenosine derivatives form triple helices with poly(U) that are analogous to those formed by non-substituted adenosine derivatives. The melting points of the complexes containing aminoadenosine derivatives and poly(U) are higher, because of an additional H-bond (Howard et al., 1966). As a result, an enhanced oligomerization was expected. However, oligomers up to trimer were produced, rather than the longer oligomers formed in the condensation of adenosine-5'-phosphoimidazolides under identical conditions.

The first true example of template-directed formation of an altered backbone was the poly(C)-directed synthesis of oligoguanylic acid catalyzed by Pb^{2+}, which produced oligomers containing primarily 2',5'-phosphodiester bonds (Sleeper and Orgel, 1979). Subsequently, template-directed synthesis of a 3',5'-pyrophosphate (Schwartz and Orgel, 1985a; Schwartz et al., 1987), a 2',5'-pyrophosphate (Visscher and Schwartz, 1987), and a 3',5'-phosphoramide backbone was achieved (Zielinski and Orgel, 1985) (Figure 10). In our laboratory it was also demonstrated that oligomers based on pyrophosphate-linked nucleotide analogues are themselves capable of functioning as templates (Visscher et al., 1989). Oligomerization of the

bisphosphoimidazolide of dG was catalyzed substantially by a pyrophosphate-linked oligomer of dC, although the reaction was less efficient than with poly(C) as template.

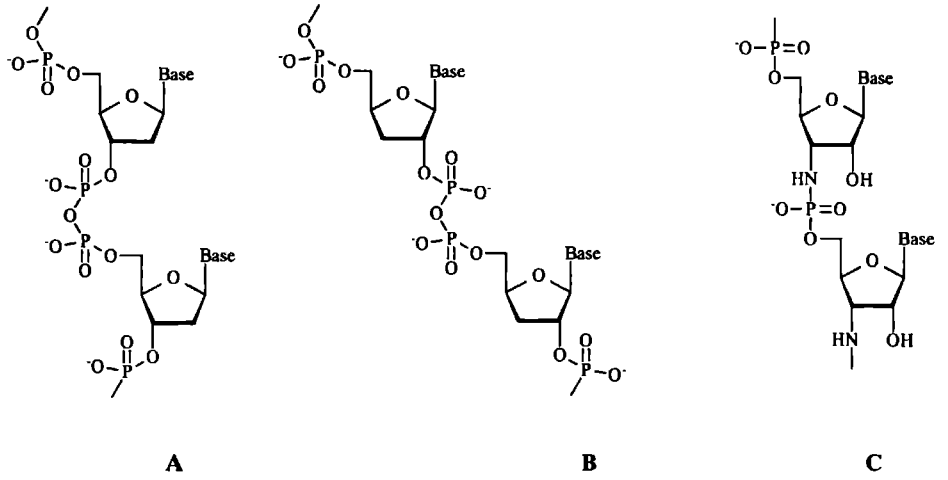


Figure 10. Alternative backbones. A, 3',5'-pyrophosphate bond; B, 2',5'-pyrophosphate bond; C, 3',5'-phosphoamide bond.

Attempts to perform template-directed reactions in which amide, ester or disulfide-linked products (Figure 11) are formed have not been successful. Oligomerization of amino acids containing the nucleotide bases adenine or uracil and using carbodiimidazole as a condensing agent were studied by Cheikh and Orgel (1990). Oligomers containing amide linkages (Figure 11) were formed in good yield, but the reaction was not catalyzed by polyuridylic acid or a complementary nucleopeptide oligomer with uracil as a base. Harada and Orgel (1990) showed that oligomerization of adenylic acid analogues (Figure 11) with a water soluble carbodiimide as condensing agent were catalyzed by uridylic acid. The α,β-unsaturated acid which possesses a trans conformation of the double bond was used, because this compound cannot cyclize. Nevertheless, the production of long oligomers based on ester linkages was inhibited by insolubility of the products. A new approach was explored by Wu and Orgel (1991). They tried to synthesize oligomers by oxidation of 3',5'-bisphosphorothioate analogues (Figure 11). Cyclization of the monomer appeared to be more efficient, inhibiting the formation of disulfide-linked oligomers.

Template-directed oligomerizations carried out with prochiral acyclic nucleotide analogues derived from glycerol and acrolein (Figure 12) were the first reactions in which the sugar residue was successfully altered. They are especially interesting as examples of possible

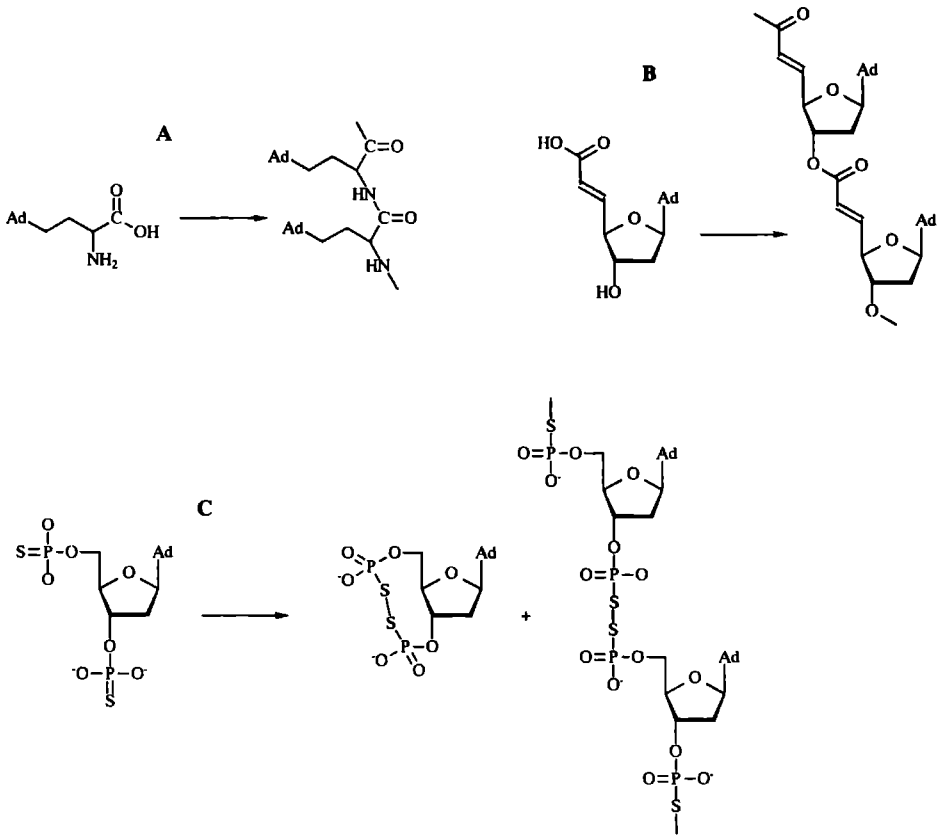


Figure 11. Formation of nucleotide analogues with different types of linkages. A, amide bond; B, ester bond; C, disulfide bonds (cyclized monomer and oligomer).

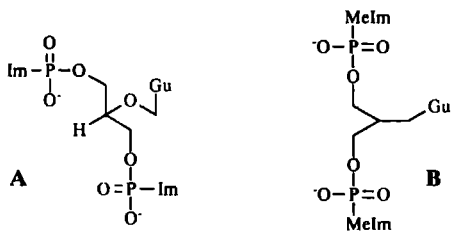


Figure 12. Structures of prochiral acyclic nucleotide analogues derived from glycerol (A) and acrolein (B).

precursors of contemporary nucleotides. Acyclic analogues of G (Figure 12) were oligomerized efficiently in the presence of a complementary polynucleotide (Visscher and Schwartz, 1988; Tohidi and Orgel, 1989; Visscher and Schwartz, 1989). However, when the template consisted of a pyrophosphate-linked, glycerol-based polynucleotide analogue, only a limited catalysis was obtained (Visscher and Schwartz, 1990b). Both the flexibility of these analogues, which leads to a considerable amount of cyclization of the monomer, as well as the atactic configuration of the oligomer, were recognized as contributing factors limiting the template-directed oligomerization.

Nucleotide analogues based on pentaerythritol.

In our laboratory the base-catalyzed condensation of formaldehyde has been reexamined under mild conditions. It was found that ultraviolet irradiation of 0.1 M formaldehyde solutions results in an unexpectedly efficient synthesis of the reduced carbohydrate pentaerythritol (compound **7** in Figure 13) (Schwartz and de Graaf, 1993). Because of the highly selective synthesis together with the symmetric structure of pentaerythritol, the hypothesis was put forward that pentaerythritol could have played a role in some primitive RNA precursor (Schwartz, 1993). It was suggested that a few simple chemical reactions might produce nonchiral nucleotide analogues as is illustrated in Figure 13 (Schwartz, 1993). After bisphosphorylation and activation, these analogues might be oligomerized to produce pyrophosphate-linked products. The analogues **10** and **11** resemble adenosine and uridine and are theoretically able to form analogous base-paired complexes with each other, although self-complementary structures are also possible. A few groups have reported this type of hydrogen bonding utilizing barbituric acid with diaminopyrimidine or related derivatives of these compounds (Lehn et al., 1990; Seto and Whitesides, 1990; Ahuja et al., 1993). However, bisphosphorylation of the analogues **10** and **11** was unsuccessful. A related achiral analogue **13** (Figure 14), with longer side chains, was found to be readily bisphosphorylated. Although no prebiotic synthesis is known for the precursor of **13** (**9**), a homologue tentatively identified as **8** has been isolated as a minor product of the irradiation of a formaldehyde solution (De Graaf, personal communication).

Independent of the question of its possible prebiotic synthesis, it is interesting to attempt to perform template-directed oligomerizations with a system completely different from RNA. The study of such a system can provide information about structural elements that are essential for nonenzymatic replication. Therefore, it was decided to extend the series of compounds to be studied to the full set of analogues shown in Figure 15.

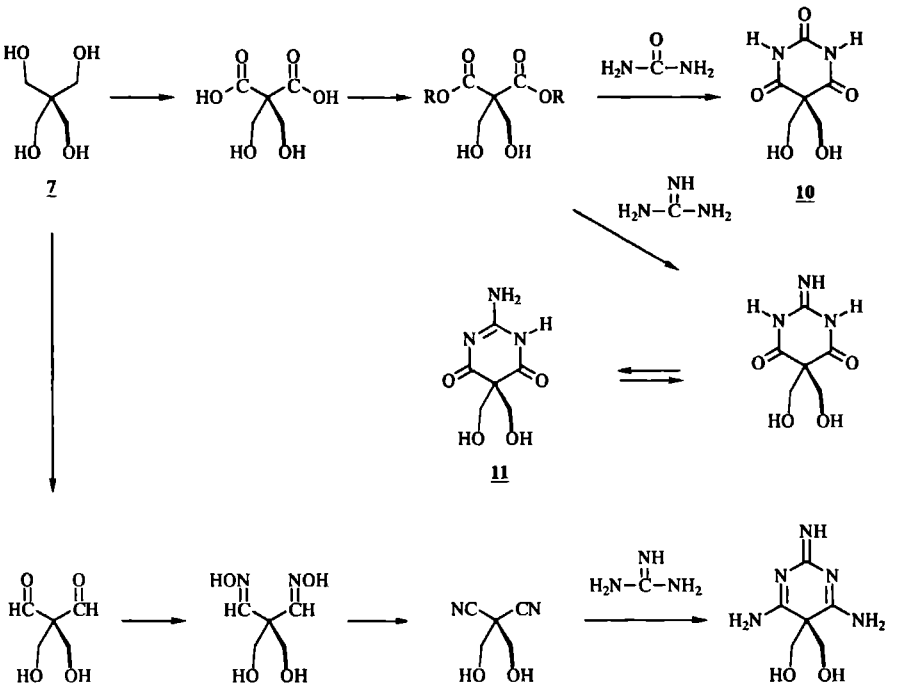


Figure 13. A speculative sequence of reactions to convert pentaerythritol into derivatives of barbituric acid (Schwartz and van Vliet, 1994)

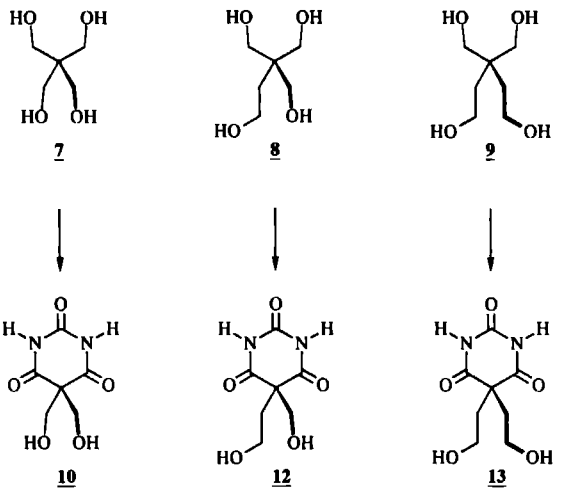


Figure 14. The relationship between structures of pentaerythritol and its homologues and the barbituric acid derivatives formed following the sequence of reactions given in Figure 13

Topics discussed in this thesis.

The aim of the investigation described in this thesis is to come to a better understanding of the properties of informational macromolecules that are important for molecular replication. We have chosen to attempt to perform the well-known nonenzymatic template-directed oligomerization developed by Orgel for mimicking this process. Instead of the naturally occurring nucleotides, the set of monomers consisting of achiral nucleotide analogues related to the pyrimidine barbituric acid, as is depicted in Figure 15, was prepared Before template-directed reactions could be performed, the properties of the nucleotide analogues, as well as the conditions for oligomerizations had to be studied. In addition, appropriate templates needed to be synthesized.

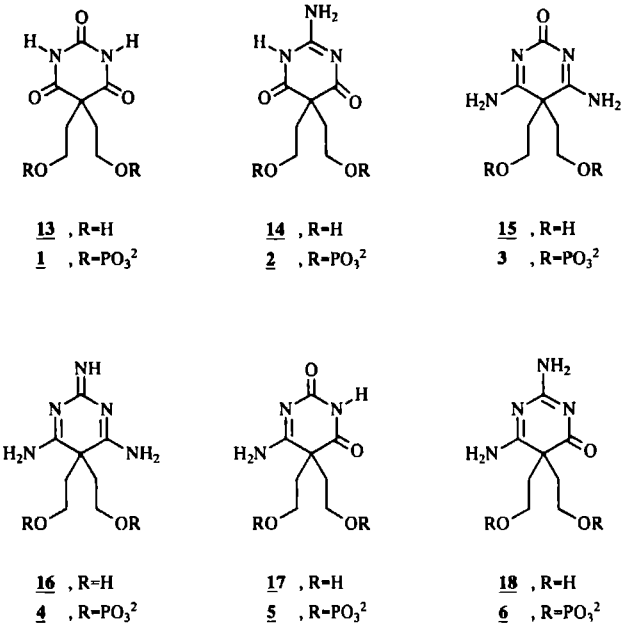


Figure 15. Structures of the acyclic nucleotide analogues used in oligomerization experiments which are described in this thesis

In Chapter 2 the synthesis of the six analogues is presented Possible conformations of some analogues are discussed The oligomerization of the activated compound **1** in aqueous solution is described in Chapter 3 The influence of divalent metal ions, pH and temperature was studied Chapter 4 describes the oligomerization of analogue **1** in the presence of the clay mineral montmorillonite In this study activation *in situ* of the bisphosphate 1 with a water soluble carbodiimide as condensing agent, as well as the preactivated bisphosphoimidazolide

of **1** were used.

Oligomerization experiments with the whole series of analogues in aqueous solution at various pH values are presented in Chapter 5. The ionization constants of the heterocyclic rings of these analogues have been determined. The influence of the ionization constants (pK values) and the number of exocyclic amino groups, which in turn is correlated with the number of nonprotonated endocyclic N-atoms, on the oligomerization reactions are discussed. Possible reaction mechanisms and an hypothesis relating the efficiency of the oligomerization to the nature of the ring are given. Chapter 6 describes the oligomerization of the analogues in mixtures of organic solvents and water. Oligomers of two potentially complementary analogues **1** and **4** were prepared and studied by UV-spectroscopy. In Chapter 7 the oligomerization of activated monomers of **4** and **6** in the presence of the complementary, pyrophosphate-linked oligomer **1** and of poly(U) was studied in mixtures of DMF and water. The role of stacking interactions and the ionization constants of the heterocyclic rings of nucleotide analogues in molecular recognition are discussed.

Synthesis of Acyclic Nucleotide Analogues: 5,5-Disubstituted Pyrimidines Related to Barbituric Acid

Abstract

Acyclic nucleotide analogues are of interest in origin of life studies. The synthesis is reported of the compounds: 5,5-di(2-phosphoethyl)-2,4,6-trioxopyrimidine (**1**), 2-amino-5,5-di-(2-phosphoethyl)-4,6-dioxopyrimidine (**2**), 4,6-diamino-5,5-di(2-phosphoethyl)-2-oxo-pyrimidine (**3**), 2,4,6-triamino-5,5-di(2-phosphoethyl)pyrimidine (**4**), 6-amino-5,5-di-(2-phosphoethyl)-2,4-dioxopyrimidine (**5**), and 2,6-diamino-5,5-di(2-phosphoethyl)-4-oxopyrimidine (**6**). These six new pyrimidine analogues were prepared by condensation of urea or guanidine with α,α-di(2-[ethoxy-1-ethoxy]ethyl)diethylmalonate (**21**), α,α-di-(2-[ethoxy-1-ethoxy]ethyl)malononitrile (**24**) or α,α-di(2-[ethoxy-1-ethoxy]ethyl)ethyl-cyanoacetate (**25**) respectively, followed by removal of the protecting groups and bisphosphorylation. Possible conformations of the analogues are discussed.

Based in part on: M.J. van Vliet, J. Visscher and A.W. Schwartz (1994) *Nucleosides & Nucleotides*, 13, 2113-2124

Introduction

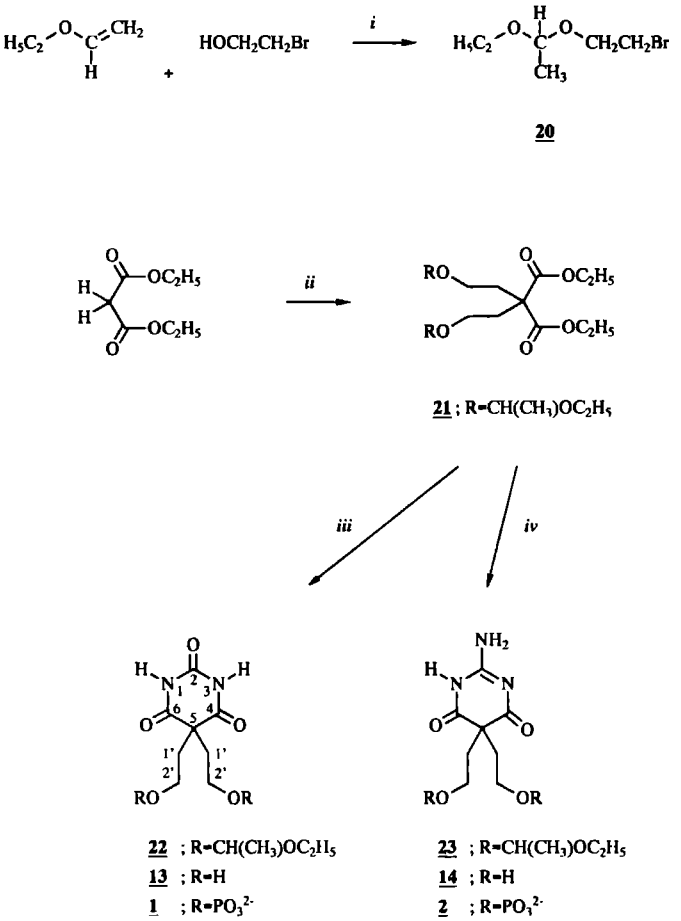
Molecular recognition via the formation of hydrogen-bonded complexes plays a central role in the design of self-replicating systems (Tjivikua et al., 1990; Nowick et al., 1991; Achilles and von Kiedrowski, 1993). Barbituric acid derivatives, which can form six hydrogen bonds with two complementary 2,4,6-triaminopyrimidine analogues, have also been utilized as the basis for a supramolecular system (Lehn et al., 1990). We have shown recently that 5,5-di(2-phosphoethyl)barbituric acid **1** can be polymerized with high efficiency in aqueous solution (van Vliet et al., 1994b). This ease of polymerization, combined with the potential for molecular recognition between barbituric acid derivatives and aminopyrimidines which are themselves derivatives of barbituric acid, suggested the possibility of studying a new model of template-directed replication based on achiral nucleotide analogues (Schwartz, 1993). The goal of this model is to test the hypothesis that nonchiral nucleotide and oligonucleotide analogues may have been precursors of the first RNA molecules (Joyce et al., 1987). The model compounds contain a pyrimidine ring related to barbituric acid, as well as a novel acyclic structural element. In order to study all possible complementary pairs involving these analogues, we have now synthesized compounds **13**, **14** (Scheme 1) and **15** to **18** (Scheme 2) as well as the phosphorylated species **1** to **6**.

Results and Discussion

Synthesis.

The compounds **13** to **18** can be viewed as being related to C-nucleosides in which an acyclic moiety is attached at C-5 of a pyrimidine ring (Chu & Suh, 1986; Doboszewski et al., 1988). To introduce the acyclic moiety at the C-5 position of the heterocyclic ring the classical malonic ester synthesis was chosen. 1-Bromo-2-(ethoxy-1-ethoxy)ethane (**20**) (Janssen & Godefroi, 1981) was used as alkylating agent. This hydroxyl protecting group is rather stable in basic conditions and is easily removed by acid hydrolysis. After bis-alkylation of the α,α-positions of diethylmalonate, malononitrile or ethylcyanoacetate the corresponding protected pyrimidine analogues were formed by condensation with urea and guanidine, respectively (Brown, 1970).

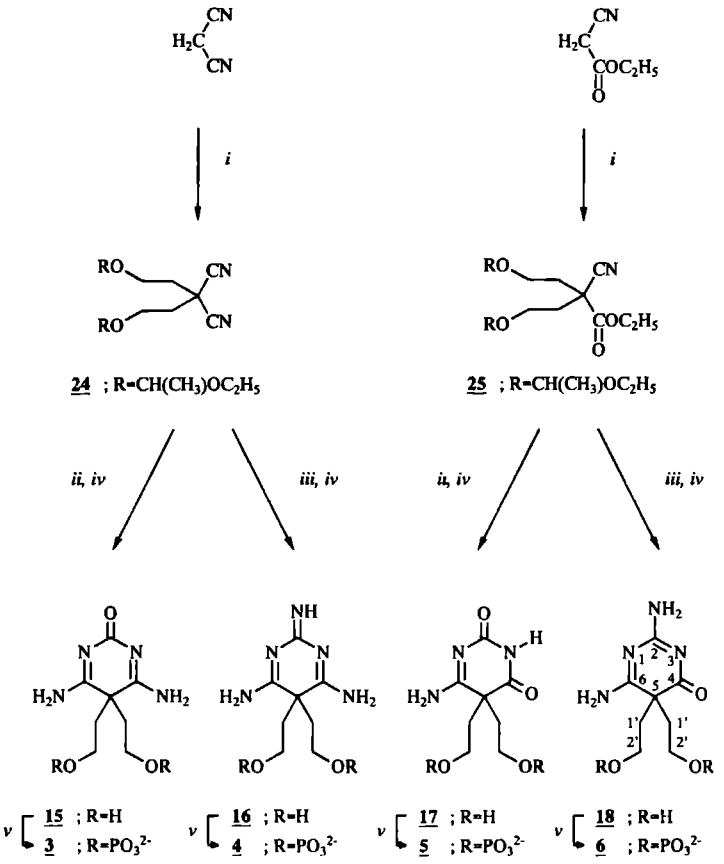
The synthesis of the analogues **1** and **2** is illustrated in Scheme 1. Diethylmalonate was treated with 2 equivalents sodium ethanolate and subsequently with 2 equivalents of the bromide (**20**) in refluxing ethanol, producing **21** in 35% yield. Compound **21** was condensed with urea in the presence of sodium ethanolate yielding 5,5-di(2-[ethoxy-1-ethoxy]-



Scheme 1. *i*, pTSOH in CH_2CH_2; *ii*, $NaOC_2H_5$ in ethanol, **20**; *iii*, urea/$NaOC_2H_5$ in ethanol; *iv*, guanidine/$NaOC_2H_5$ in ethanol.

ethyl)-2,4,6-trioxopyrimidine (**22**) in 55%. 2-Amino-5,5-di(2-[ethoxy-1-ethoxy]etyl)-4,6-dioxopyrimidine (**23**) was synthesized in 57% yield by condensation of **21** with guanidine in the presence of sodium ethanolate. Acid hydrolysis of **22** and **23** with Dowex (H^+) yielding the di(2-hydroxyethyl)-containing analogues **13** and **14** in 90-92%, which were bisphosphorylated with phosphorus oxychloride in trimethylphosphate (Slotin, 1977) to give **1** and **2** in yields of 71% and 24%.

For the synthesis of the analogues **3**, **4**, **5** and **6** almost the same approach was followed (Scheme 2). The disodium salt of malononitrile was prepared (Freeman, 1969) using



Scheme 2. *i*, NaH in DMF, **20**; *ii*, urea/$NaOC_2H_5$ in ethanol; *iii*, guanidine/$NaOC_2H_5$ in ethanol; *iv*, H^+; *v*, $POCl_3$.

NaH in dimethylformamide (DMF). DMF was preferred above dimethyl sulfoxide (DMSO) because reactions in DMSO were accompanied by major side products. The two hydroxyethyl groups, acetal protected, were introduced at the α position of malononitrile using bromide (**20**) as alkylating agent, yielding **24** in 71%. The same procedure was used for dialkylation of ethylcyanoacetate to give **25** in 77%. The compounds **24** and **25** were condensed with urea or guanidine in refluxing ethanol in the presence of sodium ethanolate, followed by the removal of the protecting groups in 1 M HCl. The overall yield of the synthesis ranged from 20% for compound **15** to 53% for compound **17**. Finally the hydroxyl groups were bisphosphorylated with phosphorus oxychloride in trimethylphosphate to give **3** to **6** in yields ranging from 60 to

87%.

Conformation.

From the ^{1}H-NMR spectra of compound **5** and **6** some information about the conformation of these two compounds can be obtained. Because of the prochiral character of the ring the protons of each methylene group are diastereotopic. This means for compound **6** that the protons of each methylene group are not equivalent, which results in a complicated ^{1}H-NMR spectrum of the ABXY-type (Günther, 1992). In the CH_2 region of this spectrum a double AB triplet and a double AB quartet can be found (Figure 1). The triplets were assigned to the H-1'-atoms and the quartets to the H-1"-atoms (Figure 2). The molecule has one stable conformation with no rotation around the C-1'-C-2' bond. The high coupling constant of -14.7 Hz in the double triplet and double quartet is due to a coupling between two sets of geminal H-atoms (H-1' and H-1"). In the triplets the coupling constant is 4.7 Hz indicating a preference for *gauche* relationships between H-1' and H-2" and between H-1' and H-2'. The vicinal coupling constants of the quartets are 5.0 and 8.4 Hz, indicating a preference for a *gauche* relationship between H-1" and H-2" and an *anti* relationship between H-1" and H-2'. From these coupling constants a preference appears for the orientation depicted in I or II (Figure 2). One of these conformers is probably stabilized by intramolecular electrostatic interactions or H-bonds between the phosphate groups and substituents on the ring (pK= 6.9; Chapter 5).

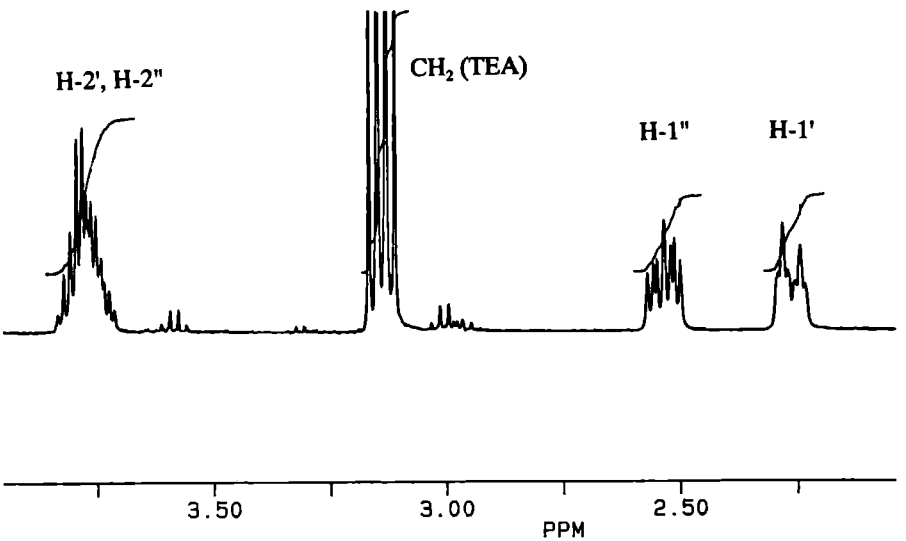


Figure 1. ^{1}H-NMR spectrum of compound **6** as a triethylammonium salt.

From molecular modeling studies and the ^{1}H-NMR results it was concluded that the H-1' and H-1" protons of this molecule would be oriented *trans* to the ring so that repulsion between the acyclic chains is minimized, as illustrated in projection III (Figure 2). Because of the prochiral character of the ring, a symmetric relationship between the acyclic chains seems logical. This assumption together with the results of NMR and molecular modeling resulted in two possible conformations (A or B in projection III).

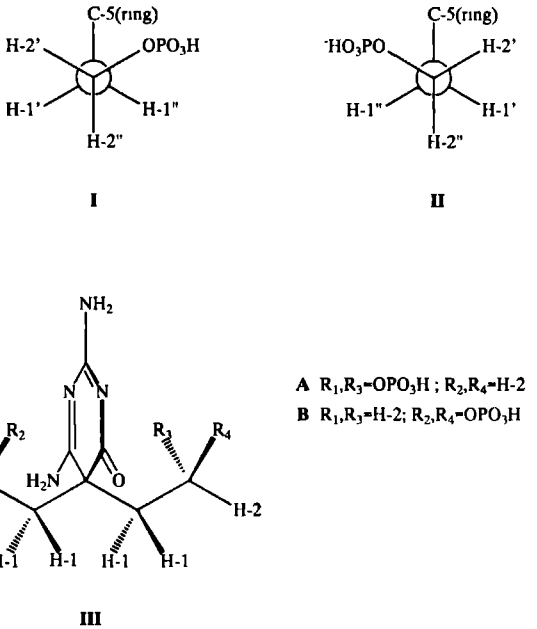


Figure 2. Projections I and II (along a C-1'–C-2' axis); two possible orientations of compound **6** suggested by the coupling constant of the ^{1}H-NMR spectrum. Projection III; two possible conformations of compound **6** suggested by ^{1}H-NMR results and molecular modeling studies. It should be noted that one of the endocyclic N-atoms may be protonated (pK=6.9, Chapter 5).

In the CH_2 region of the ^{1}H-NMR spectrum of compound **5** an extra triplet is observed if compared with compound **6** (Figure 3). This extra triplet is assigned to the H-1' and H-1"-atoms. Irradiation at the frequency of H-2'/H-2" produced only a singlet and two doublets with a geminal coupling constant of -14.4 Hz. An explanation could be that for some protons the diastereotopic effect is neutralized. The reason for this effect is unknown, however, since the ionization constants of the heterocyclic ring of both compounds are different (Chapter 5), charge differences by protonation of the ring (Günther, 1992) may play a role.

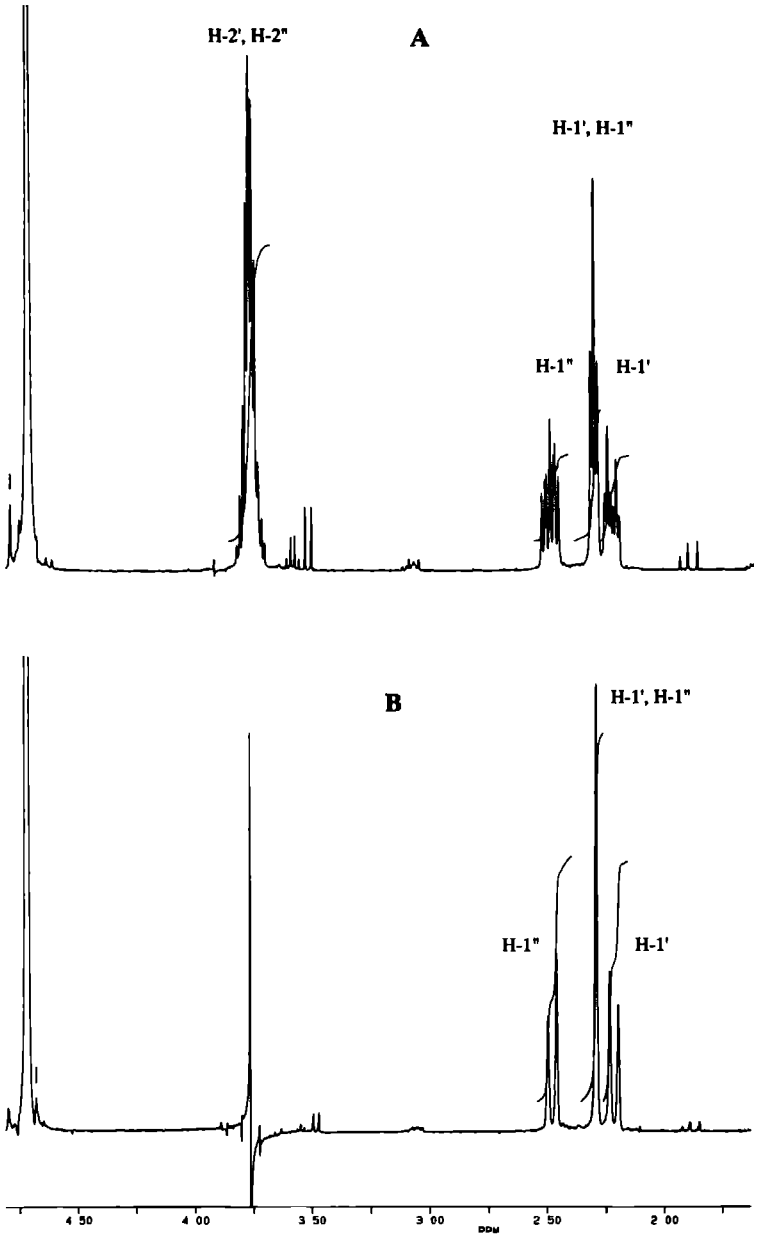


Figure 3. A; ^{1}H-NMR spectrum of compound **5** as a Na^+-salt. B; ^{1}H-NMR spectrum of compound **5** irradiated at the H-2'/H-2" position.

Because of the achiral character of the other four bisphosphorylated compounds, the protons of each methylene group are equivalent and no information about the conformation of these analogues can be obtained from their ^{1}H-NMR spectra.

Experimental

Proton nuclear magnetic resonance spectra were recorded with a Varian EM 390 or Bruker 400 MHz spectrometer, ^{31}P-NMR spectra (proton decoupled) with a Bruker 400 MHz spectrometer. Infrared spectra were recorded with a Perkin-Elmer 457 spectrophotometer, ultraviolet spectra with a Beckman DU-40 spectrophotometer using cells of 10 mm pathlength. Mass spectra were recorded on a 7070 E VG Analytical mass spectrometer using methane as chemical ionization gas at 70 ev. HPLC analyses were performed on a LiChrospher RP-18 (5 μm) column (Merck) in 0.02 M KH_2PO_4 with a linear gradient of methanol (0-6% over 12 min.) at a flow rate of 1 ml/min. Peak detection was by absorbance monitoring at 254 nm. Q-Sepharose (Fast Flow), DEAE-Sephadex (A25) and alkaline phosphatase (type III from *Escherichia coli*) were purchased from Pharmacia; Dowex AG1-X2(Cl^-) and Dowex A6 50W-X8(H^+) from Bio-Rad Laboratories. Triethylammonium bicarbonate (TEAB) buffer was made by bubbling carbon dioxide gas through a stirred mixture of triethylamine (TEA, 825 ml) and water (1175 ml) at 0°C until the pH of the clear solution was 7.5. All solvents were distilled from appropriate drying agents.

1-Bromo-2-(ethoxy-1-ethoxy)ethane **20**.
Ethyl vinyl ether (85 ml, 0.89 mol) was added to a stirred solution of 2-bromoethanol (84.6 g, 0.68 mol) and pyridinium *p*-toluenesulphonate (1.2 g) in 200 ml CH_2Cl_2 at 0°C. After 1 h the solution was washed with water, dried ($MgSO_4$) and concentrated under reduced pressure. The resulting oil was purified by distillation under reduced pressure to give product **20** in a yield of 86% (115 g, 0.58 mol).
Bp: 70-73°C at 20 mm Hg. IR (neat): 2880, 2960, 1100, 1375. ^{1}H-NMR ($CDCl_3$, TMS) δ ppm: 1.1-1.4 (t+d, 6H, 2×CH_3, J_t=7.2 Hz, J_d=5.3 Hz), 3.3-4.0 (m, 6H, 3×CH_2), 4.7-4.8 (q, 1H, CH, J=5.4 Hz).

α,α-Di(2-[ethoxy-1-ethoxy]ethyl)diethylmalonate **21**.
To a solution of sodium ethanolate (0.2 mol), freshly prepared from 4.6 g Na in 150 ml anhydrous ethanol, was added diethylmalonate (16 g, 0.1 mol) at 55°C. After reacting for 15 minutes, 1-bromo-2-(ethoxy-1-ethoxy)ethane **20** (40 g, 0.2 mol) was added dropwise. The

resulting mixture was refluxed for 20 h, filtered and concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 and washed with water. The organic phase was dried over $MgSO_4$, filtered and evaporated. Purification was by distillation *in vacuo*. Yield: 13.68 g (34.8 mmol, 35%) (monosubstituted product yield 32%).
Bp: 127-132°C at 0.4 mm Hg. IR (neat): 2950, 1730, 1480, 1020, 980, 850. ^{1}H-NMR ($CDCl_3$, TMS) δ ppm: 1.3 (m, 18H, 6×CH_3), 2.3 (t, 4H, 2×CH_2), 3.6 (m, 8H, 4×OCH_2), 4.2 (q, 4H, 2×CO_2CH_2), 4.7 (q, 2H, 2×CH).

General synthesis of compounds **22** *and* **23**.
To a solution of sodium ethanolate (40 mmol), prepared from 0.93 g sodium in 25 ml anhydrous ethanol, was added α,α-di(2-[ethoxy-1-ethoxy]ethyl)diethylmalonate **21** (3.92 g, 10 mmol), followed by urea (0.73 g, 11 mmol) or guanidine.HCl (1.05 g, 11 mmol). The solvent was evaporated in 4 h at 110°C. The residue was dissolved in water (100 ml) and adjusted to pH 6 with 6 M HCl. The precipitate produced was collected by filtration, washed with water en dried under vacuum.

5,5-Di(2-[ethoxy-1-ethoxy]ethyl)-2,4,6-trioxopyrimidine **22**.
Yield: 55%. Mp: 125-127°C. IR (KBr): 3150, 2900, 1700. ^{1}H-NMR (CD_3OD, external TMS) δ ppm: 1.2 (2×t, 12H, 4×CH_3), 2.2 (t, 4H, 2×CH_2), 3.5 (m, 8H, 4×OCH_2), 4.6 (q, 2H, 2×CH).

2-Amino-5,5-di(2-[ethoxy-1-ethoxy]ethyl)-4,6-dioxopyrimidine **23**.
Yield: 57%. Mp: 210°C (decomposition). IR (KBr): 3300, 3000, 2750, 1600 (br). ^{1}H-NMR (DMSO-d_6, external TMS); 1.1 (2×t, 12H, 4×CH_3), 2.3 (t, 4H, 2×CH_2), 3.4 (m, 8H, 4×OCH_2), 4.5 (q, 2H, 2×CH), 6.9 (s (br), 3H, NH_2+NH).

General procedure for the deprotection of compounds **22** *and* **23**.
To a suspension of 5,5-di(2-[ethoxy-1-ethoxy]ethyl)-2,4,6-trioxopyrimidine **22** (1 mmol) or 2-amino-5,5-di(2-[ethoxy-1-ethoxy]ethyl)-4,6-dioxopyrimidine **23** (1 mmol) in water (1 ml) Dowex A6 50W-X8(H^+) was added. The suspension was stirred for 15 minutes until a clear solution was formed. The solution was heated - to prevent precipitation of **13** or **14** - and filtered, followed by evaporation under reduced pressure. The products were purified by recrystallization in water.

5,5-Di(2-hydroxyethyl)-2,4,6-trioxopyrimidine **13**.
Yield: 90%. Mp: 162-167°C. IR (KBr): 3380, 3100, 2900, 1700. ^{1}H-NMR (D_2O + NaOD, external TMS) δ ppm: 2.0 (t, 4H, 2×CH_2), 2.9 (t, 4H, 2×OCH_2). UV (H_2O) λ (nm) (ε):

λ_{max}=238.5 (6500) in 0.1 M NaOH. MS, m/z (rel. int.): 217 (50.1), M^++H; 199 (46.9), M^++H-H_2O; 157 (100), M^++H-OH-NCOH. Anal. Calcd. for $C_8H_{12}N_2O_5$: C, 44.45; H, 5.59; N, 12.96. Found: C, 44.58; H, 5.46; N, 12.63.

2-Amino-5,5-di(2-hydroxyethyl)-4,6-dioxopyrimidine **14**.
Yield: 92%. Mp: 210°C (decomposition). IR (KBr): 3300, 3000, 2800, 1590. ^{1}H-NMR (D_2O + NaOD, external TMS) δ ppm: 1.8 (t, 4H, 2×CH_2), 2.8 (t, 4H, 2×OCH_2). UV (H_2O) λ (nm): λ_{max}=266.5 (15500) in 0.1 M NaOH, λ_{max}=257.5 (21000) in 0.1 M HCl. MS, m/z (rel. int.): 215 (25), M^+; 199 (48.7), M^+-NH_2; 168 (57.9), M^+-NH_2-CH_2OH; 157 (100), M^+-NH_2-NCO; 156 (56.6), M^+-NH_2-NCOH. Anal. Calcd for $C_8H_{13}N_3O_4$: C, 44.65; H, 6.09; N, 19.52. Found: C, 44.95; H, 6.36; N, 19.59.

α, α-Di(2-[ethoxy-1-ethoxy]ethyl)malononitrile **24**.
To a stirred suspension of NaH (1.42 g, 59.2 mmol) in 20 ml anhydrous dimethylformamide (DMF) a solution of malononitrile (1.98 g, 30 mmol) in 15 ml DMF was added dropwise at 0°C. After 15 minutes 1-bromo-2-(ethoxy-1-ethoxy)ethane **20** (15 g, 76 mmol) was added dropwise at 0°C, and the reaction mixture was stirred overnight at room temperature. The solvent was evaporated *in vacuo*, then the residue was dissolved in CH_2Cl_2 (200 ml) and washed twice with saturated sodium bicarbonate (75 ml). The organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The oil was purified by short path distillation *in vacuo* to give 6.4 g (21.4 mmol, yield 71%) of **24**.
Bp: 94-100°C at 0.4 mm Hg. IR (neat): 2900, 2270, 1400, 1100, 950. ^{1}H-NMR ($CDCl_3$, TMS) δ ppm: 1.2-1.4 (m, 12H, 4×CH_3), 2.2-2.4 (t, 4H, 2×CH_2, J=6.0 Hz), 3.3-4.1 (m, 8H, 4×CH_2O), 4.6-4.8 (q, 2H, 2×CH, J=5.3 Hz).

α, α-Di(2-[ethoxy-1-ethoxy]ethyl)ethylcyanoacetate **25**.
A mixture of ethylcyanoacetate (3.16 g, 28 mmol) in 10 ml anhydrous DMF was added dropwise to a stirred suspension of NaH (1.49 g, 62 mmol) in 20 ml anhydrous DMF at 0°C. After 30 minutes, during which time the NaH dissolves, 1-bromo-2-(ethoxy-1-ethoxy)ethane **20** (15 g, 76 mmol) was added dropwise at 0°C. The suspension produced was stirred for 3 h at room temperature. The solvent was evaporated under reduced pressure and the precipitate was partitioned between CH_2Cl_2 and water. The organic layer was washed with saturated sodium bicarbonate, dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. Purification was by short path distillation *in vacuo* to give **25** as a colorless oil in 77% yield (7.45 g, 21.6 mmol).
Bp: 110-116°C at 0.4 mm Hg. IR (neat): 2900, 2250, 1740, 1380, 1240, 1100, 940. ^{1}H-NMR

($CDCl_3$, TMS) δ ppm: 1.2-1.5 (m, 15H, 5×CH_3), 1.8-2.6 (m, 4H, 2×CH_2), 3.3-4.0 (m, 8H, 4×OCH_2), 4.1-4.4 (q, 2H, CO_2CH_2, J=7.4 Hz), 4.6-4.8 (q, 2H, 2×CH, J=5.3 Hz).

General synthesis of compounds **15**, **16**, **17**, *and* **18**.

To a refluxing solution of sodium ethanolate (10.8 mmol), prepared from 0.25 g sodium in 40 ml anhydrous ethanol, was added α,α-di(2-[ethoxy-1-ethoxy]ethyl)malononitrile **24** (2.0 g, 6.7 mmol), or α,α-di(2-[ethoxy-1-ethoxy]ethyl)ethylcyanoacetate **25** (2.0 g, 5.8 mmol) followed by urea (0.44 g, 7.3 mmol) or guanidine.HCl (0.69 g, 7.3 mmol). After 4 h the mixture was neutralized with 6 M HCl and partitioned between CH_2Cl_2 and water. The organic layer was washed with water, dried ($MgSO_4$) and evaporated to dryness. The hydroxyl protecting groups were hydrolyzed by treating the residue with 1 M HCl (20 ml) for 5 min. HCl was removed by evaporation *in vacuo* and treating with Dowex AG1-X2(OH^-). Purification was by recrystallization in ethanol.

4,6-Diamino-5,5-di(2-hydroxyethyl)-2-oxopyrimidine **15**.

Yield; 410 mg (1.9 mmol, 28%), ^{1}H-NMR (D_2O, external TMS) δ ppm: 2.1-2.2 (t, 4H, 2×CH_2, J=6.6 Hz), 3.3-3.4 (t, 4H, 2×OCH_2, J=6.6 Hz). UV (H_2O) λ (nm) (ε): λ_{max}=227.5 (18260), 265 (6560) in 0.1 M NaOH, λ_{max}=254 (8550) in 0.1 M HCl. MS, m/z (rel. int.): 198 (100), M^+-NH_2; 167 (27.2), $M^+-NH_2-CH_2OH$; 155 (53.5), M^+-NH_2-NCOH. Anal. Calcd. for $C_8H_{14}N_4O_3$. 0.69 HCl: C, 40.18; H, 6.19; N, 23.42. Found: C, 40.79; H, 6.19; N, 23.08.

2,4,6-Triamino-5,5-di(2-hydroxyethyl)pyrimidine **16**.

Yield: 850 mg (4.0 mmol, 60%), ^{1}H-NMR (D_2O + NaOD, external TMS) δ ppm: 2.1-2.2 (t, 4H, 2×CH2, J=7.5 Hz), 3.2-3.5 (t, 4H, 2×OCH_2, J=7.5 Hz). UV (H_2O) λ (nm) (ε): λ_{max}=231.5 (15500), 281 (6310) in 0.1 M NaOH, λ_{max}=239.5 (18200), 278.5 (7790) in 0.1 M HCl. MS, m/z (rel. int.): 197 (97.3), M^+-NH_2; 166 (18.3), $M^+-NH_2-CH_2OH$; 155 (28.8), $M^+-NH_2-NCNH_2$. Anal. Calcd. for $C_8H_{14}N_5O_2$. 1.45 HCl: C, 36.34; H, 5.89; N, 26.48. Found: C, 37.07; H, 6.24; N, 25.78.

6-Amino-5,5-di(2-hydroxyethyl)-2,4-dioxopyrimidine **17**.

Yield: 0.860 g (4.0 mmol, 69%), ^{1}H-NMR (D_2O , external TMS) δ ppm: 2.2-2.7 (m, 4H, 2×CH_2), 3.6-3.7 (t, 4H, 2×OCH_2, J=6.0 Hz). UV (H_2O) λ (nm) (ε): λ_{max}=253.0 (6270) in 0.1 M NaOH. MS, m/z (rel. int.): 199 (100), M^+-NH_2; 168 (27.1) $M^+-NH_2-CH_2OH$; 156 (64.9) M^+-NH_2-CONH. Anal. Calcd. for $C_8H_{13}N_3O_4$. 0.35 HCl: C, 42.15; H, 5.90; N, 18.43. Found: C, 42.74; H, 6.03; N, 18.15.

2,6-Diamino-5,5-di(2-hydroxyethyl)-4-oxopyrimidine **18**

Yield: 0.490 g (2.3 mmol, 40%), ^{1}H-NMR (D_2O + NaOD, external TMS) δ ppm: 2.0-2.7 (m, 4H, 2×CH_2), 3.5-3.6 (t, 4H, 2×OCH_2, J=5.3 Hz). UV (H_2O) λ (nm) (ϵ): λ_{max}=230 (17000), 268.5 (7460) in 0.1 M NaOH, λ_{max}=257.5 (12500) in 0.1 M HCl. MS. m/z (rel. int.): 198 (100), M^+-NH_2; 167 (18.6), $M^+-NH_2-CH_2OH$; 156 (63.6), $M^+-NH_2-NCNH_2$. Anal. Calcd. for $C_8H_{14}N_4O_3$. 1.57 HCl: C, 35.45; H, 5.80; N, 20.67. Found: C, 35.31; H, 6.18; N, 20.75.

General procedure for the phosphorylation of compounds **13** to **18**.

To a suspension of 100 mg of each of the compounds in 2 ml trimethylphosphate, 0.25 ml phosphorus oxychloride was added dropwise at 0°C. The mixture was stirred for 2 hours at 0°C, during which time a clear solution was formed. To the reacting mixture was added 30 ml of a cold solution of triethylamine bicarbonate (TEAB, 2 M, pH 8) at -20°C. The solvent was evaporated under reduced pressure. Purification was by column chromatography on DEAE-Sephadex using a TEAB gradient of 0.05 M to 0.5 M at pH 8. The products were obtained as triethylammonium salts and the yields were determined by UV.

5,5-Di(2-phosphoethyl)-2,4,6-trioxopyrimidine **1**

The procedure for the synthesis was as described above with a small modification. After addition of the phosphorus oxychloride, the reaction mixture was stirred for 3 h at 0°C followed by 1 h at room temperature.

Yield: 71%. ^{31}P-NMR (D_2O, external trimethylphosphate) δ ppm: -0.330 (s). ^{1}H-NMR (D_2O, external TMS) δ ppm: 2.302-2.330 (t, 4H, 2×CH_2, J=5.5 Hz), 3.775-3.815 (q, 4H, 2×OCH_2, J=5.4 Hz). UV (H_2O) λ (nm): λ_{max}=238 in 0.1 M NaOH.

2-Amino-5,5-di(2-phosphoethyl)-4,6-dioxopyrimidine **2**.

Yield: 24%. ^{31}P-NMR (D_2O, external trimethylphosphate) δ ppm: 0.909 (s). ^{1}H-NMR (D_2O, external TMS) δ ppm: 2.503-2.540 (t, 4H, 2×CH_2, J=7.3 Hz), 3.742-3.797 (q, 4H, 2×OCH_2, J=7.3 Hz). UV (H_2O) λ (nm): λ_{max}=266.5 in 0.1 M NaOH, λ_{max}=263.0 in 0.1 M HCl.

4,6-Diamino-5,5-di(2-phosphoethyl)-2-oxopyrimidine **3**.

Yield: 60%. ^{31}P-NMR (D_2O, external trimethylphosphate) δ ppm: -0.3387 (s). ^{1}H-NMR (D_2O, external TMS) δ ppm: 2.552-2.579 (t, 4H, 2×CH_2, J=5.3 Hz), 3.843-3.883 (q, 4H, 2×OCH_2, J=5.4 Hz). UV (H_2O) λ (nm): λ_{max}=226.5, 264.5 in 0.1 M NaOH, λ_{max}=254.0 in 0.1 M HCl.

2,4,6-Triamino-5,5-di(2-phosphoethyl)pyrimidine **4.**

Yield. 81%. ^{31}P-NMR (D_2O, external trimethylphosphate) δ ppm: 0.0282 (s). ^{1}H-NMR (D_2O, external TMS) δ ppm: 2.441-2.470 (t, 4H, 2×CH_2, J=5.8 Hz), 3.743-3.786 (q, 4H, 2×OCH_2, J=5.8 Hz). UV (H_2O) λ (nm): λ_{max}=238, 279 in 0.1 M NaOH, λ_{max}=238.5, 276.5 in 0.1 M HCl.

6-Amino-5,5-di(2-phosphoethyl)-2,4-dioxopyrimidine **5.**

Before NMR the triethylammonium salt of compound **5** was converted into a sodium salt on Dowex A6 50W-X-8 (Na^+ form).

Yield. 87%. ^{31}P-NMR (D_2O, external trimethylphosphate) δ ppm. -0.1677 (s). ^{1}H-NMR (D_2O, external TMS) δ ppm. 2.209-2.257 (2×t, 1.14H, 1.14×CH-1', $J_{H1H1'}$=-14 4 Hz, J_{H1H2}=$J_{H1H2'}$=4 6 Hz), 2.288-2.316 (t, 1.71H, 0 86×CH-1' + 0.86×CH-1", $J_{H1,H2}$=5.7 Hz), 2.454-2.525 (2×dd, 1 14H, 1.14×CH-1", $J_{H1'H1}$=-14.4 Hz, $J_{H1'H2}$=8.3 and $J_{H1'H2'}$=5.8 Hz), 3 737-3 814 (m, 4H, 2×(H-2'+H-2")) UV (H_2O) λ (nm): λ_{max}=250 in 0 1 M NaOH.

2,6-Diamino-5,5-di(2-phosphoethyl)-4-oxopyrimidine **6.**

Yield 72%. ^{31}P-NMR (D_2O, external trimethylphosphate) δ ppm: -0 0160 (s). ^{1}H-NMR (D_2O, external TMS) δ ppm: 2.232-2.289 (2×t, 2H, 2×CH-1', $J_{H1H1'}$=-14 8 Hz, $J_{H1H2'}$=$J_{H1H2'}$=4.7 Hz), 2 498-2.568 (2×q, 2H, 2×CH-1", $J_{H1'H1}$=-14.8 Hz, $J_{H1',H2}$=8.4 and $J_{H1'H2'}$=5 0 Hz), 3 714-3.832 (m, 4H, 2×(H-2'+H-2")) UV (H_2O) λ (nm): λ_{max}=231.5, 269 in 0.1 M NaOH, λ_{max}=258.5 in 0.1 M HCl.

General treatment of compounds **1** to **6** *with alkaline phosphatase.*

The compounds (0 05 μmol) were treated with 0 1 unit of alkaline phosphatase in 100 μl Tris-HCl (0 04 M, pH 8) containing 0 02 M $MgCl_2$ at 37°C Samples were taken after 5, 15, 60 and 120 minutes and analyzed by HPLC on a C_{18} reverse phase column. Degradation of the bisphosphates produced, sequentially, the monophosphates and the dephosphorylated compounds

Molecular modeling.

Molecular mechanics studies were performed using the AMBER force field from the Hyperchem program (Autodesk, Inc 1993, 2320 Marinship Way, Sausalito, CA 94965, USA). No solvent molecules were included. The torsion along the C-1'-C-2' bond was restrained to 60°, as determined from the coupling constants of the ^{1}H-NMR spectra The torsions along the C-5-C-1' bond were changed in 30° increments, and the energy of the structure was minimized each time. Two series of minimizations were conducted: one with sodium counterions

and one without counterions.

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An Achiral (Oligo)Nucleotide Analogue

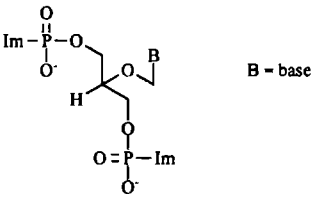
Abstract

An achiral nucleoside analogue based on barbituric acid has been synthesized. The analogue, which is 5,5-di(2-phosphoethyl) barbituric acid, undergoes extensive oligomerization in aqueous solution, when activated, to produce pyrophosphate-linked chains. In contrast to a number of other bisphosphorylated nucleoside analogues which have been studied, the compound has little tendency to cyclize. The possible prebiotic implications are discussed.

Based in part on: M.J. van Vliet, J. Visscher and A.W. Schwartz (1994) *J. Mol. Evol.* 38, 438-442

Introduction

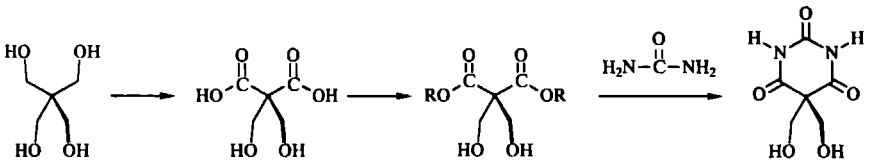
During the past several years, a number of publications have drawn attention to the problems which the prebiotic synthesis of ribose introduces into the discussion of the origins of the first RNA molecules (Joyce et al., 1987; Shapiro, 1988). These concerns fall into two general types: (1) the lack of a plausible prebiotic synthesis which produces significant yields of ribose, as opposed to all possible carbohydrates (Reid and Orgel, 1967; Shapiro, 1988; Schwartz and de Graaf, 1993) and (2) the chiral nature of ribose, which is responsible for inhibition of the template-directed oligomerization of mononucleotides (Joyce et al., 1984). Nucleotide analogues in which ribose has been replaced by a "prochiral" structure based on glycerol have been studied, primarily because of the second of these concerns (Schwartz and Orgel, 1985a; Visscher and Schwartz, 1988; Schneider and Benner, 1990). We have investigated the oligomerization of the analogue ImpBpIm:



In the absence of a template, the oligomerization (which produces pyrophosphate-linked chains) is severely restricted by the tendency of the activated monomer to form a cyclic pyrophosphate. Although oligomerization is sharply improved in the presence of polycytidylic acid (when B = guanine), a glycerol-based, pyrophosphate-linked, synthetic oligomer of pCp was less effective as template (Visscher and Schwartz, 1990b). Under the best conditions found, in the presence of $MnCl_2$, approximately 70% of the monomer still cyclized. Both cyclization of the monomer and the atactic nature of the template were recognized as serious problems (Visscher and Schwartz, 1990b; Rodriguez and Orgel, 1991). These results have led us to seek alternative solutions to the "ribose problem".

Recently, we have shown that the reduced carbohydrate pentaerythritol is formed with great specificity by uv irradiation of 0.1 M aqueous formaldehyde (Schwartz and de Graaf, 1993). The symmetrically substituted carbon atom in this compound attracted our attention and suggested the possibility that achiral nucleotide analogues might be prepared from pentaerythritol or related structures. Elsewhere we have argued that a small number of simple chemical steps could convert pentaerythritol into an achiral derivative of barbituric acid, **10** in

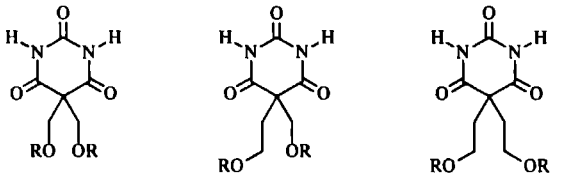
Figure 1 (Schwartz, 1993)[1].



10

Figure 1. A speculative reaction scheme showing the possible prebiotic formation of the barbituric acid derivative **10** from pentaerythritol via oxidation, activation, and condensation with urea (Schwartz, 1993).

Variations of this sequence of reactions would produce a suite of barbituric acid derivatives which could partake in the formation of supramolecular structures, based on either self-complementary or Watson-Crick-type hydrogen bond formation (Schwartz, 1993). In order to carry out a first test of this proposal, we have synthesized compound **10** as well as its homologue, compound **13**. Since the latter compound proved to be more easily phosphorylated than **10**, we report here the results of the oligomerization of this bisphosphoimidazolide in aqueous solution (Figure 2).



10 ; R=H
19 ; R=PO_3H^-

12 ; R=H

13 ; R=H
1 ; R=PO_3H^-
1a ; R=PO_2^-Im

Figure 2. The structures of nucleoside analogues based on barbituric acid.

[1]The distinction between achiral and prochiral is not trivial. ImpBpIm does not display optical activity because two of the substituents on the central carbon atom are identical. However, it is prochiral since there is only one plane of symmetry and consequently the central C- H can rotate between two orientations. If these become fixed in a polymer, the chirality of the polymer will depend upon the sequence of orientations. The analogue **19** has two planes of symmetry through the central carbon atom. It is achiral since rotating either of these planes 180° produces identical configurations.

Materials and Methods

Alkaline phosphatase (type III from *Escherichia coli*), and pyrophosphatase (type II from *Crotalus adamanteus* venom) were purchased from Sigma. Phosphodiesterase I from *C. adamanteus* venom was from Pharmacia P-L Biochemicals. ^{1}H-NMR and ^{31}P-NMR spectra were recorded with a Bruker 400 MHz spectrometer. Chemical shifts (δ) are given in ppm relative to TMS (^{1}H-NMR) and to trimethylphosphate (^{31}P-NMR) in $CDCl_3$ as external standards. Triethylammonium bicarbonate (TEAB) buffer was made by bubbling carbon dioxide gas through a stirred mixture of triethylamine (TEA, 825 ml) and water (1175 ml) at 0°C until the pH of the clear solution was 7.5.

HPLC.

The purity of the products (**13**, **1** and **1a**) was controlled by HPLC using a C_{18} reverse-phase column (Merck) in a K_2HPO_4 buffer (0.02 M, pH 8.5) with a linear gradient (0-20%) of methanol-water (3:2). The flow rate was 1.0 ml/min and peak detection was monitored at 254 nm. Analysis of the oligomerization products was carried out on an RPC-5 column (anion exchange) in 0.02 M NaOH with a linear gradient of $NaClO_4$ (0-0.02 M over 30 min) at a flow rate of 1.0 ml/min. The eluent was monitored at 240 nm. The RPC-5 system employs Adogen 464, a trialkylmethylammonium chloride, with the predominant chain length of the alkyl groups being C_8 - C_{10}, on hydrophobic polychlorotrifluoroethylene resin with a particle size < 35 μm (Pearson et al., 1971).

Synthesis.

Compound **13** (5,5-di(2-hydroxyethyl)-2,4,6-trioxopyrimidine) was synthesized as described by Visscher and Schwartz (1993) (Chapter 2). Phosphorylation was achieved by a modification of the procedure described by Visscher and Schwartz (1988). The analogue (260 mg, 1.2 mmol) was suspended in dry trimethylphosphate (6 ml) and cooled to 0°C. Under argon, phosphorylchloride (1.1 ml, 12 mmol) was added dropwise in 10 min, during which a clear solution was formed. The reaction mixture was stirred for 3 h at 0°C followed by 1 h at room temperature. The reaction mixture was then treated with a solution of cold TEAB (100 ml, 1 M, pH 8.0) and stirred for another 15 min. After evaporation under vacuum, the crude reaction product was purified on a DEAE-Sephadex column (A25, Pharmacia) with a linear gradient of TEAB (0.05-0.5 M, pH 8.0). The fractions containing the desired material were evaporated to dryness and coevaporated with ethanol (2×25 ml). The bisphosphate of **13** (**1**) was obtained as a triethylammonium salt. The yield, as determined by UV (0.1 N NaOH; λ_{max}=238nm; ϵ=6500), was 760 μmol (64%). The identity of compound **1** was confirmed by

treatment with alkaline phosphatase, monophosphorylated **13** and **13** being produced sequentially. ^{1}H-NMR (D_2O): δ ppm: 2.302-2.330 (t, 4H, 2×CH_2); 3.775-3.815 (q, 4H, 2×CH_2). ^{31}P-NMR (D_2O): δ ppm: -0.330 (s).

Phosphoimidazolide **1a** was prepared by a modification of a standard method (Joyce et al., 1984). An anhydrous solution of imidazole (89 mg, 1.3 mmol), triphenylphosphine (352 mg, 1.3 mmol) and 2,2'-dithiodipyridine (288 mg, 1.3 mmol) in DMF (1.8 ml) and TEA (250 μl) was added dropwise to a suspension of the phosphate **1** (0.3 mmol) in DMSO (1.25 ml) and TEA (200 μl). The reaction mixture was shaken for a few minutes until a clear solution was formed, which was stirred for 2 h under argon at room temperature. The product was precipitated by adding an anhydrous mixture of acetone (50 ml), diethylether (50 ml), TEA (4 ml) and a saturated solution of $NaClO_4$ in acetone (0.5 ml). The suspension was centrifuged, the supernatant was removed, and the product was washed three times with an anhydrous mixture of acetone-diethylether (50:50) and finally twice with anhydrous diethylether. After drying under vacuum, product **1a** was obtained as a white powder (123 mg, 258 μmol, 79%). ^{31}P-NMR (DMSO-d_6): δ ppm: -8.314 (s). ^{1}H-NMR (DMSO-d_6) δ ppm: 1.869-1.902 (t, 4H, 2×CH_2, J=5.16 Hz), 3.368 (s (br), 2×OCH_2), 6.803, 6.967, 7.513 (3×s, 6H, 6×CH (imidazole)). The identity of product **1a** was confirmed by HPLC after hydrolysis with sodium acetate.

Oligomerizations.

All reactions were carried out in 1.5 ml polyethylene tubes (Eppendorf) in a total volume of 10 μl. To each tube aqueous solutions of $MnCl_2$, $MgCl_2$ or $CdCl_2$ were added as required, followed by NaCl, and the tubes were centrifuged and evaporated to dryness. Imidazole-HCl buffer (1.0 M, pH 6.5, 4μl) followed by a freshly prepared solution of the phosphoimidazolide **1a** was added at 0°C. The reaction mixtures were mixed thoroughly, centrifuged and incubated at 1°C or 37°C. Reactions were quenched by addition of two equivalents of KEDTA per divalent metal ion, diluted to 100 μl, and stored at -25°C. For HPLC-analysis aliquots with a total theoretical monomer content of 0.05 μmol were taken from the mixtures. Surviving imidazolides were hydrolyzed by incubation with sodium acetate buffer (pH 4.0, 100 μl, 0.1 M) for 1 h at 50°C.

Enzyme digestions.

For alkaline phosphatase digestion, samples (0.05 μmol monomer equivalent) were treated with Tris-HCl (0.04 M, pH 8, 100 μl) containing 0.02 M $MgCl_2$ and 0.1 units of enzyme for 4 h at 37°C. For phosphodiesterase I digestion, samples were treated with Tris-HCl (0.2 M, pH 9.0, 100 μl) containing 0.04 M $MgCl_2$ and 0.2 units of enzyme. Incubation was as above. For

pyrophosphatase digestion, samples were treated with Tris-HCl (0.2 M, pH 7.2, 100 μl) containing 0.04 M $MgCl_2$ and 0.2 units of enzyme. Incubation was for 16 h at 37°C. After addition of KEDTA (1.0 M, 4.0 μl) the mixtures were analyzed by HPLC.

$ZrCl_4$ treatment.

Cleavage of the pyrophosphate linkages with $ZrCl_4$ was performed according to Visscher and Schwartz (1992). A sample of an isolated oligomer (120 μl, 0.03 ODU measured at 240 nm) was added to a solution of sodium acetate (NaAc) (3.0 M, pH 5.0, 20 μl) containing $ZrCl_4$ (1.0 M, 5 μl). After incubation at 50°C for various times the reaction mixture was quenched with KEDTA (1.0 M, 15 μl), neutralized with NaOH (10 M, 10 μl) and filtered. Analysis was carried out on an RPC-5 column.

Results

Table 1. Product distributions in the oligomerization of compound **1a**[a].

Incubation temp. and time		Monomer	Relative yield of oligomers of length n (%)[c]					
		(%)[b]	$n>2$	$n\geq3$	$n\geq4$	$n>5$	$n\geq10$	$n\geq15$
1°C	1week	75	25	6	1.0	0.2		
	7weeks	35	65	37	18	9	0.2	
	13weeks	22	78	54	32	19	1.4	0.1
37°C	3days	16	84	62	37	24	2.5	0.2

[a] Conditions: 0.05 M bisphosphoimidazolide **1a**; 0.4 M $MnCl_2$; 0.1 M NaCl; 0.4 M Imidazole-HCl (pH 6.5).
[b] Includes <5% of cyclic monomer.
[c] Includes cyclic oligomers (total<15%).

Oligomerization of compound **1a** produced an extensive series of linear oligomers, as well as a subset of short cyclic oligomers (Figure 3). The products of the oligomerization were characterized by isolating them from the RPC-5 column and studying the products produced by enzymatic and chemical hydrolysis. In contrast to the glycerol-based analogues previously described (Visscher and Schwartz, 1988), oligomers of **1** were found to be resistant to the action of venom phosphodiesterase. They could, however, be hydrolyzed slowly with venom pyrophosphatase: a pentamer, for example, producing tetramer, trimer, dimer and monomer in

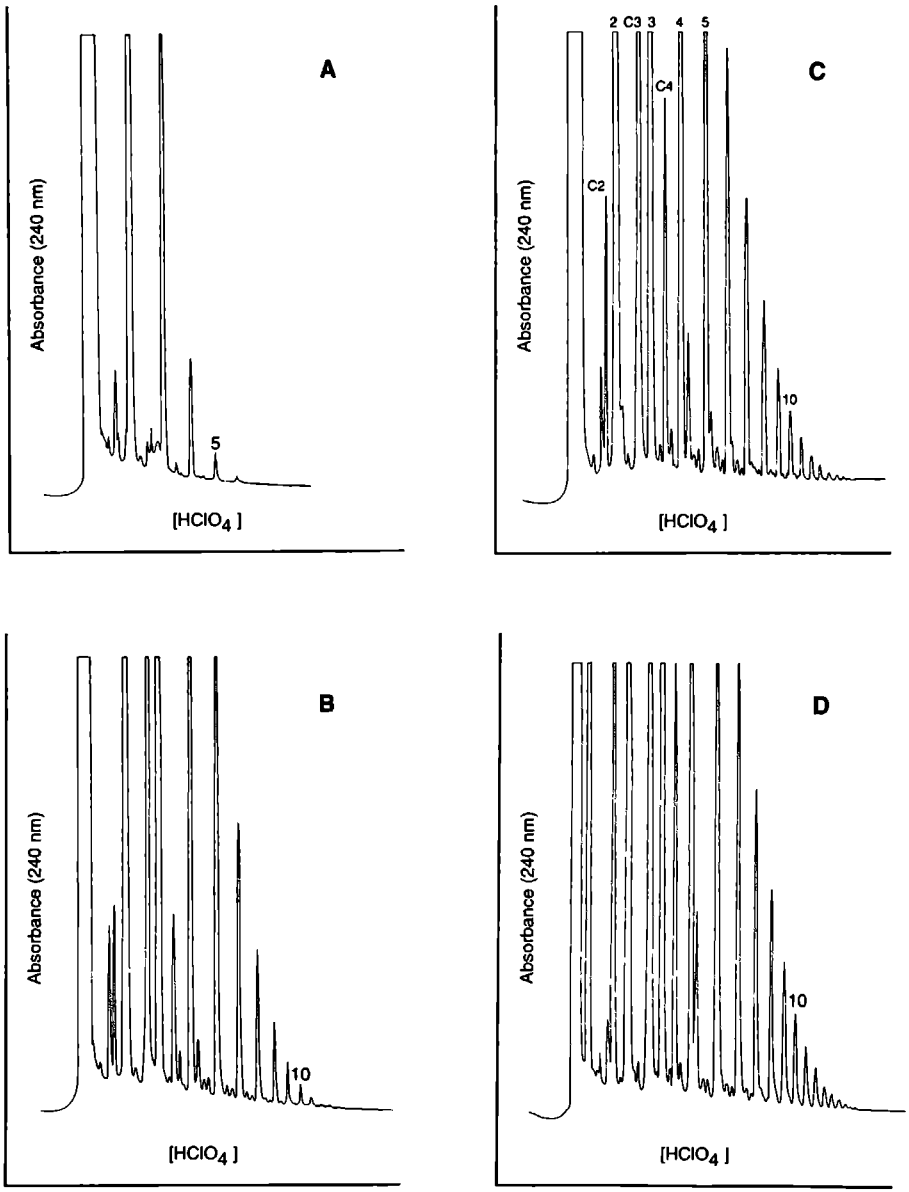


Figure 3. Oligomerization of compound **1a** (0.05 M) in the presence of 0.4 M $MnCl_2$, 0.1 M NaCl, and 0.4 M imidazole-HCl (pH 6.5). **A-C**, 1°C for 1, 7, and 13 weeks. **D**, 37°C for 3 days. Conditions for HPLC on RPC-5: elution with a linear $NaClO_4$ gradient (pH 12, 0-0.02 M, 30 min) at a flow rate of 1.0 ml/min. UV absorption was monitored at 240 nm. The numbers indicate the chain lengths of linear oligomers. The peaks labelled with C2, C3 and C4 correspond to cyclic dimer, trimer and tetramer.

increasing proportions with time. The length of the pentamer was also established by hydrolysis in the presence of $ZrCl_4$, which produced the expected series of products (Visscher and Schwartz, 1992). Cyclic oligomers where shown to be resistant to alkaline phosphatase and were degraded via the corresponding linear oligomers in the presence of $ZrCl_4$

The oligomerization at 37°C was complete within 3 days, after which time no remaining imidazolides were detectable. The reaction at 1°C required 13 weeks in the presence of Mn(II). The reaction with Mg(II) was still incomplete at this time. Comparison of the distributions of products at several times at the two temperatures (Figure 3 and Table 1) suggests that the ultimate results at both temperatures are comparable. Of the cyclic oligomers formed, the trimer was most abundant (see below).

The extent of oligomerization at both 1°C and 37°C was found to depend strongly on the nature as well as on the concentration of the divalent metal ion. Figure 4 compares the results of three oligomerizations carried out under identical conditions, but in the presence of Mn(II), Mg(II), or Cd(II) In the presence of Mn(II), which was the most efficient metal

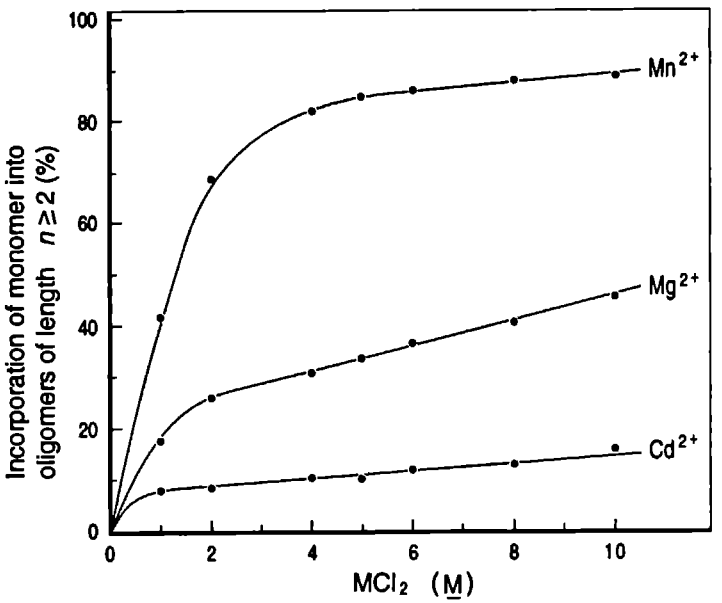


Figure 4. Effects of divalent metal ions on the oligomerization of compound **1a** (0 1 M) in the presence of 0 1 M NaCl and 0 4 M imidazole-HCl (pH 6 5) at 1°C for 5 weeks Results are plotted as the total yields of oligomers ($n > 2$) against concentration of metal chloride

tested, significant oligomerization was observed at monomer concentrations as low as 0.005 M (at 37°C for 3 days). Within the monomer concentration range studied (0.005 to 0.1 M), the degree of oligomerization increased with divalent metal ion concentration for all three metals (Figure 4). The formation of cyclic oligomers was also greater at higher Mn(II) or Mg(II) concentrations. In the presence of 0.4 M $MnCl_2$ (0.05 M monomer, 37°C, 3 days) 12.5% cyclic trimer was formed. NaCl concentrations were less critical, but had a positive effect on overall oligomerization yields up to 1.0 M NaCl.

The pH optimum for the reaction was 6.0, although substantial oligomerization (55% conversion of monomer for Mn(II) and 30% for Mg(II) at 37°C) was observed even at pH 8.0 (Figure 5).

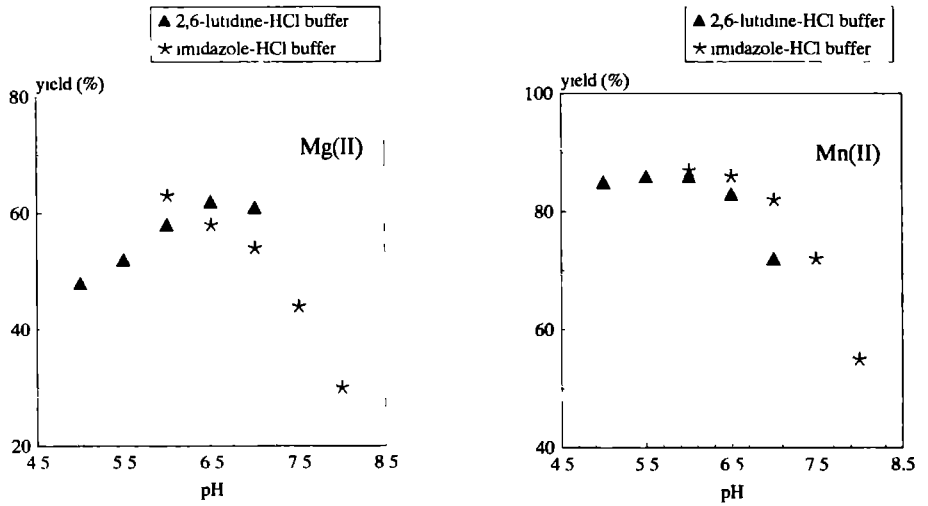


Figure 5. Influence of the pH on the yield of oligomers of length $n \geq 2$ in the reaction of compound **1a** in the presence of Mg(II) (left diagram) and Mn(II) (right diagram). Conditions: 0.05 M monomer **1a**, 0.4 M MCl_2, 0.1 M NaCl, and 0.4 M buffer; incubated at 37°C for 3 days.

Discussion

Oligomerization of compound **1a** is surprisingly efficient. Under conditions of high monomer concentration (0.1 M) and in the presence of 0.4 M $MnCl_2$, more than 90% of the monomer is converted to oligomers, with chain lengths exceeding 20 (not shown). These results far exceed, both in chain length and yield, previous results obtained in the oligomerization of nucleotide analogues in the absence of a template (Schwartz et al., 1987; Visscher and Schwartz,

1989). Even at concentrations as low as 0.005 M, significant oligomerization was observed (41%). An important factor in the reaction is the very restricted tendency of the monomer to cyclize. This is in marked contrast to the ease of formation of the eight-membered cyclic pyrophosphates of glycerol-1,3- and ribose-3,5- bisphosphates (Visscher and Schwartz, 1988; Rodriguez and Orgel, 1991). In previous studies of the effect of Mn(II) on oligomerizations of pyrimidine-containing 2'-deoxynucleoside bisphosphates (Visscher and Schwartz, 1989), it was found that the effect of the metal was to increase the extent of oligomerization without affecting the degree of cyclization of the monomer. This observation also applies to the oligomerization of compound **1a**. In one set of experiments carried out at a monomer concentration of 0.05 M (at 37°C), replacing Mg(II) with Mn(II) resulted in an increase of the total extent of the oligomerization from 55 to 84%, while the extent of monomer cyclization remained at 4%. The highly restricted internal pyrophosphate formation of compound **1** may be explained partially by the observation that 10-membered systems generally are unfavorable with respect to efficient ring closure, given their position between the opposing influences of ring strain and entropy effects (Illuminati and Mandolini, 1981). A second factor which may also be of importance in this regard is the relatively rigid relationship of the substituents at C-5 of barbituric acid.

The exceptional activity of Mn(II) in catalyzing pyrophosphate bond formation confirms earlier observations (Visscher and Schwartz, 1989). Mn(II) parallels the affinity of Mg(II) for one oxygen donor group, but Mn(II) has a higher affinity than Mg(II) for complexes in which more than one O-donor groups and, especially, nitrogen groups are involved (Fraústo da Silva and Williams, 1991). This suggests that the formation of a complex in which phosphate and phosphoimidazolide groups are brought into proximity is more favorable with Mn(II) than with Mg(II). Another important factor for the difference in catalysis may be the difference in lability (kinetic factor) between manganese and magnesium complexes (Cotton and Wilkinson, 1976). For example, the exchange of water is approximately 10^2 times slower from around Mg^{2+} than from around Mn^{2+}. The ease of transition between the six-and seven-coordinate states presumably accounts for the lability of Mn(II) complexes compared with those of Mg(II) (Fraústo da Silva and Williams, 1991). Raising the concentration of divalent metal ions resulted in an increase in the extent of oligomerization (Figure 4). In addition to the possible specific role of Mn(II) referred to above, metal ions can increase the reactivity of the phosphoimidazolides either directly or through a bound base, e.g., hydroxide. Several hydrolytic and synthesizing enzymes have various metal ions at the active site, for example, zinc and magnesium in alkaline phosphatase and three zinc ions in phospholipase C (Hough, et al., 1989). Some bacterial catalases found in thermophiles or lactobacilli even posses a dimanganese active site (Pessiki et al., 1994). Model catalysts have also been used to show the great

enhancements of reactivity of two metal ions at active sites over one (Fraústo da Silva and Williams, 1991).

The role of Cd(II) in these reactions is somewhat less clear. Sawai et al. (1992) have studied the metal-ion-catalyzed formation of dinucleoside pyrophosphates from the reactions of mononucleotides with mononucleotide imidazolides. They observed strong catalysis with Cd(II) in reactions between purines and a lower effect for pyrimidines. Our own (unpublished) experiments confirm the activity of Cd(II) in the oligomerization of the guanine-containing analogue ImpGpIm. However, this metal has a marginal effect on the oligomerization of compound **1a**. In contrast to the observation of Sawai et al. (1992) that the catalytic effect of Mn(II) on AppA formation is maximal at an equimolar ratio of mononucleotide imidazolide to metal, we have found a sharp increase in activity with much higher levels of Mn(II) (Figure 4). However, no direct comparison between these results is possible, since the nature of the interaction (involving two phosphoimidazolide groups versus a phosphoimidazolide plus a phosphate), the structures (barbituric acid versus adenine), as well as the reaction itself (oligomerization versus single bond formation) are all different.

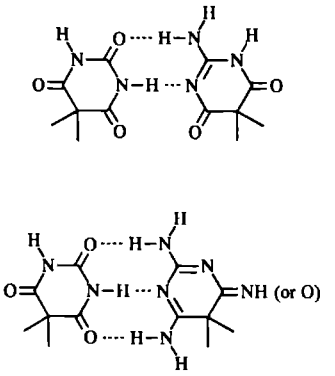


Figure 6. Some examples of possible hydrogen-bonded pairs between **1** or **19** and related barbituric acid-based analogues. The pairs shown are analogous to uracil-adenine (top) and cytosine-guanine (bottom) base pairs (Schwartz, 1993).

Prebiotic Implications

This research is part of a program the goal of which is to develop models for the prebiotic synthesis and replication of the first nucleic-acid-like molecules. Oligomers of either analogue **19** or **1** are potential templates for oligomerizations of related achiral analogues which are

capable of forming complementary pairs, such as those illustrated in Figure 6. The ease of oligomerization of achiral analogue **1a** is therefore of intrinsic interest, independent of the question of its possible derivation from pentaerythritol. However, a homologue tentatively identified as a precursor of compound **12** has been isolated as a minor product of the irradiation of formaldehyde solutions, suggesting that a reasonably prebiotic synthesis of analogue **13** may also be attainable (De Graaf, unpublished results). The possibility of carrying out template-directed oligomerizations based on structures such as those shown in Figure 6 will be the subject of future work.

Acknowledgements.
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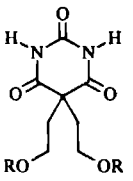
Montmorillonite catalysis of the oligomerization of a nucleotide analogue related to barbituric acid

Abstract

The bisphosphate **1** was oligomerized in aqueous solution by addition of a water-soluble carbodiimide as condensating agent. In the presence of Na^+-montmorillonite, pyrophosphate-linked oligomers up to the pentamer were produced, while in the absence of this clay mineral only a small amount of dimer was formed. The reaction was also stimulated by the presence of divalent cations, such as Mg(II) and Mn(II). Imidazole enhanced the reaction and caused a change in the product distribution. The oligomerization of the bisphosphoimidazolide **1a** was also catalyzed by the presence of Na^+-montmorillonite.

Introduction

As described in Chapter 3, oligomerization of analogue **1a** (Figure 1) produces an extensive series of pyrophosphate-linked oligomers in aqueous solution (van Vliet et al., 1994b). Although reactions with a monomer concentration of 0.1 M yield oligomers with a chain length of 20, at lower concentrations (0.005 M) only short oligomers up to the trimer are formed. Since it is unlikely that high concentrations of nucleotides or their analogues would have been present on the prebiotic earth, it is appropriate to consider how such compounds might have been concentrated from dilute solution. Possible mechanisms which have been suggested include concentration on polypeptides (Barbier et al., 1994), mineral surfaces (Gibbs et al., 1980; Schwartz and Orgel, 1985b; Acevedo and Orgel, 1986), and clay minerals (for example, Schwartz et al., 1987; Ferris and Ertem, 1992a). Ferris and coworkers have investigated extensively the role of montmorillonite in binding and promoting the oligomerization of nucleotides under dilute conditions (0.015 M monomer). They observed that phosphodiester bond formation between ribonucleotides and deoxyribonucleotides was catalyzed by certain montmorillonite clays, when a water soluble carbodiimide (EDAC) was used as activating agent (Ferris and Kamaluddin, 1989; Ferris et al., 1990). A more efficient production of oligomers was observed when a phosphoimidazolide of a nucleoside was used as a preactivated nucleotide (Ferris and Ertem, 1992b). However, when the monomers consist of pyrimidines the products formed are rather short oligomers with a maximum length of approximately 3.



1 ; R=PO_3H^-
1a ; R=PO_2^-Im

Figure 1. The structure of 5,5-di(2-phosphoethyl)-2,4,6-trioxopyrimidine (compound **1**) and its activated analogue 5,5-di(2-imidazolylphosphoethyl)-2,4,6-trioxopyrimidine (compound **1a**).

In general, pyrophosphate bond formation is more efficient than the condensation of activated nucleotides to produce phosphodiesters (Rodriquez and Orgel, 1991). Studies performed with 3',5'-bisphosphoimidazolides of guanosine showed that the oligomerization was unaffected by several minerals, clay minerals and polypeptides (Schwartz et al., 1987). In the present study we report the effect of Na^+-montmorillonite on the oligomerization of the bisphosphate **1** with EDAC as condensating agent as well as the preactivated bisphospho-

imidazolide **1a**.

Background

Montmorillonite is a dioctahedral smectite mineral with the ideal empirical formula $Si_4Al_2O_{10}(OH)_2$. However, all naturally occurring montmorillonites contain other metal ions such as Mg^{2+}, Fe^{2+} and Fe^{3+} substituted in the Al^{3+} positions or Al^{3+} substituted in the Si^{4+} positions. The substitution by cations with a lower electrostatic positive charge results in an excess negative charge on the montmorillonite lattice which is neutralized by associated cations such as Na^+, K^+, Ca^{2+} and Mg^{2+}. Montmorillonite has a layered structure composed of two tetrahedral silicate sheets and a central octahedral alumina sheet (Figure 2). The layers are able to stack with exchangeable cations and water molecules occupying the space between the layers (Newman, 1990). Negatively charged organics, such as nucleotide analogues, can bind to the exchangeable cations in the interstices and on the surface of the layers. On the other hand, positively charged organics, such as EDAC, can replace the exchangeable cations. The layer edges contain positive charges due to hydrated Al^{3+}-groups, which are not coordinated to the bulk montmorillonite structure. Phosphate groups of nucleotide analogues may bind to the edges by displacement of the water bound to the Al^{3+}. Water coordinated to the edges of montmorillonite is also a source of acidity. Another factor contributing to the acidity are solvated cations associated with the negatively charged surface layer.

$$M^{m+}(H_2O)_n = M^{m+}(H_2O)_{n-1}(OH)^- + H^+ \quad (1)$$

In both instances the acidity is a result of dissociation of water molecules (equation 1) strongly polarized by the positively changed metal ions or lattice-bound Al^{3+} (Ferris, 1993).

Experimental

Montmorillonite 22A was a gift from J.P. Ferris. The clay mineral was ground to a coarse powder with a mortar and pestle and converted to its Na^+-form by the saturation method (Lawless et al., 1985). EDAC was purchased from Janssen Chimica. Fresh solutions of EDAC were prepared at 0°C. The bisphosphate **1** and the bisphosphoimidazolide **1a** were synthesized as described earlier (van Vliet et al., 1994a, 1994b; Chapters 2 and 3). Ultraviolet spectra were recorded with a Beckman DU-40 spectrophotometer. Measurements of pH were made using pH indicator paper (Fluka AG) with pH range 5.4-7.0 and 6.4-8.0. HPLC-analysis of oligomers was carried out on an RPC-5 column in 0.02 M NaOH with a linear gradient of

$NaClO_4$ (0 to 0.02 M over 30 min) at 1.0 ml/min. The eluent was monitored at 240 nm.

MONTMORILLONITE

Composition

Tetrahedral	Octahedral	
(Si_4)	(Al_2)	$O_{10}(OH)_2$
$(Si_{3.89}Al_{0.12})$	$(Al_{1.40}Fe_{0.32}Mg_{0.31})$	$O_{10}(OH)_2$

Structure

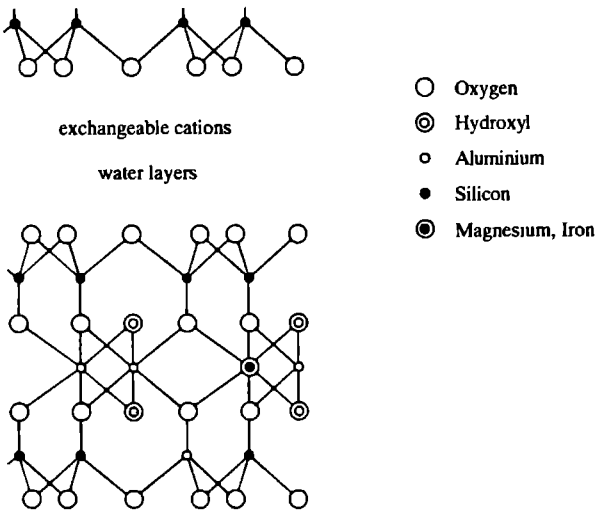


Figure 2. The composition and structure of Montmorillonite (Theng, 1974).

Adsorption of the bisphosphate **1** *on Na^+-montmorillonite 22A.*

To a 1.5 ml polyethylene tube with 10 mg Na^+-montmorillonite was added a solution (200 μl) containing 0.0055 M of compound **1** and 0.2 M NaCl. The contents were mixed and incubated for 24 h at 20°C. The mixture was then centrifuged and 30 μl of the solution was diluted for UV spectral measurements. The extent of adsorption of compound **1** to the montmorillonite was determined at pH 12 by the decrease in absorbance when compared with the absorbance of an identical dilution of the stock solution.

Oligomerization of the bisphosphate **1** *with EDAC on Na^+-montmorillonite 22A.*
All reactions were performed in 1.5 ml polyethylene tubes in a total volume of 200 μl. To the tubes was added a solution of 0.11 M compound **1** (10 μl), 1.0 M NaCl (40 μl) and, when required, 1.0 M $MgCl_2$ (20 μl) or 1.0 M $MnCl_2$ (20 μl), followed by addition of water to bring the volume to 180 μl. The montmorillonite (10 mg) was added, the contents were mixed and centrifuged, and the mixtures were incubated for 24 h at 20°C. Then an ice-cold 0.5 M solution of EDAC (20 μl) was added to the mixtures, the pH was measured, and the mixtures were incubated at 20°C. Control experiments which did not contain montmorillonite were performed under the same conditions. Reactions were also conducted in 0.1 M imidazole-HCl buffer (pH 6.5). After incubation for 5 days the pH of the mixtures was measured. In general the pH of the mixtures containing imidazole-HCl buffer (pH 6.5±0.1) was a little higher than the pH of the mixtures without this buffer (pH 6.1±0.2). The reaction mixtures without montmorillonite were quenched by addition of 1.0 M KEDTA (50 μl). The mixtures with montmorillonite were centrifuged and the supernatants were removed. To the residues was added a solution (100 μl) containing 0.25 M $K_4P_2O_7$ and 0.25 M KEDTA (pH 9.0), the contents were resuspended and were allowed to stand at room temperature for 20 minutes. The mixture was then centrifuged for 1 minute and the supernatant was removed. This procedure was repeated and the three extractions were combined. For HPLC-analysis aliquots with a total theoretical monomer content of 0.055 μmol were taken from the mixtures, diluted to 4.0 ml and filtered through a 0.45 μm Milipore filter.

Reactions were also conducted with 0.01 M analogue **1** under the same conditions as described above. Activation was by addition of 1.0 M EDAC (20 μl). After 3 days a second solution of EDAC (5.0 μl, 4.0 M) was added to some of the mixtures. After incubation for, in total, 7 days, the mixtures were treated as described above.

Oligomerization reactions of the bisphosphoimidazolide **1a** *on Na^+-montmorillonite 22A.*
All reactions were performed in 1.5 ml polyethylene tubes in a total volume of 200 μl. To the tubes was added 20 mg Na^+-montmorillonite 22A, 1.0 M NaCl (40 μl) and, when required, 1.0 M $MgCl_2$ (20 μl) or $MnCl_2$ (20 μl). After addition of water to bring the volume to 180 μl, the contents were mixed and centrifuged. Finally, a freshly prepared ice-cold solution of compound **1a** (0.1 M, 20 μl) was added to the mixtures, the contents were mixed carefully and incubated at 20°C for 7 days. Control experiments which did not contain montmorillonite were performed under the same conditions. Reactions were also performed under the same conditions but in the presence of 0.1 M imidazole-HCl buffer (pH 6.5). The reaction mixtures without montmorillonite were quenched by addition of 1.0 M KEDTA (50 μl). Before HPLC-analysis surviving imidazolides were hydrolyzed by incubation with 0.1 M sodium acetate

solution (pH 4.0) for 1 h at 50°C. The reaction mixtures with montmorillonite were quenched and treated as described above.

Results and Discussion

Oligomerization of compound **1** *with EDAC on Na^+-montmorillonite 22A.*

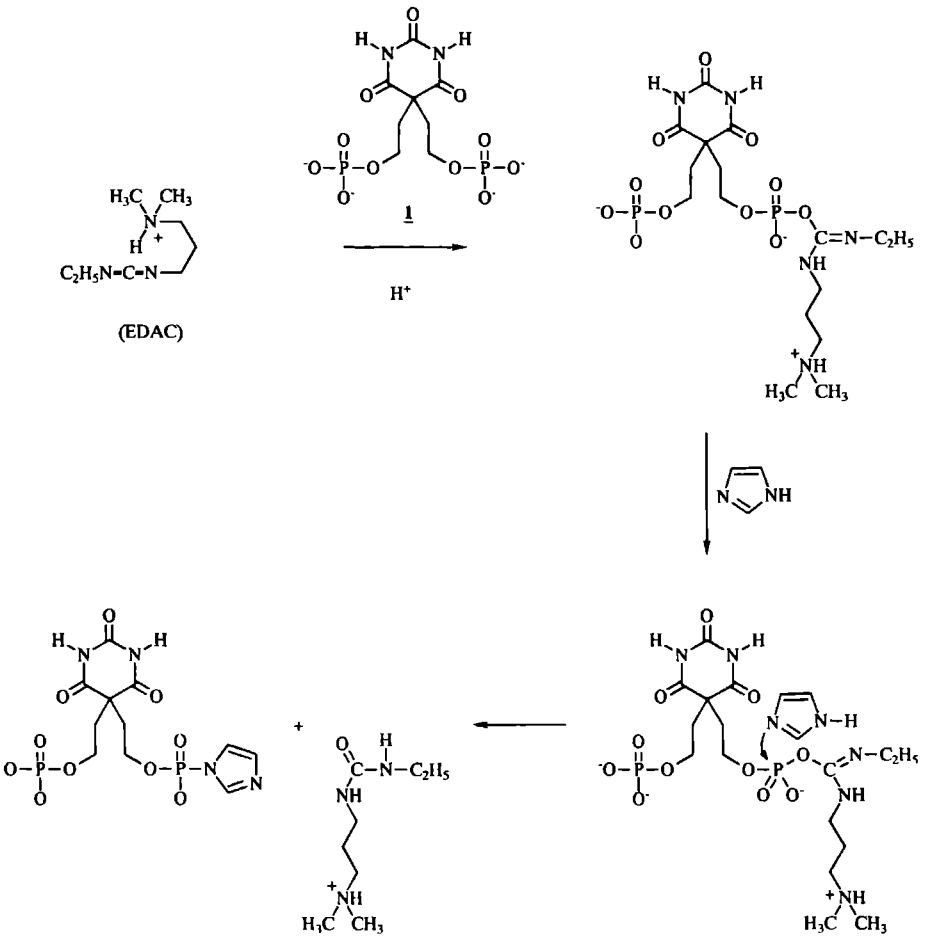
UV measurements showed that under the conditions described in the experimental section 34% of compound **1** was bound to Na^+-montmorillonite 22A in the presence of 0.2 M NaCl. From reported data it is known that $MgCl_2$ and $MnCl_2$ enhance the absorption of nucleotides to montmorillonite (Ferris and Kamaluddin, 1989; Kawamura and Ferris, 1994). EDAC is also partially adsorpted on the mineral surface (Ferris et al., 1989).

Table 1. Oligomerization reactions of 0.0055 M bisphosphate **1** with EDAC in the presence of Na^+-montmorillonite 22A.[a] Effect of 5 equivalents EDAC per phosphate group.

Mont	M(II)	Im[b]	M (%)[c]	Relative yield of oligomers of length *n* (%)			
				$n \geq 2$	$n \geq 3$	$n \geq 4$	$n \geq 5$
-	-	-	99	1.4			
-	Mg	-	98	2.2			
-	Mn	-	98	1.8			
+	-	-	93	7	0.3		
+	Mg	-	92	9	0.5		
+	Mn	-	87	13	1.5	0.2	
-	-	+	95	4.9	0.2		
-	Mg	+	93	7	0.1		
-	Mn	+	76	24	2.4	0.4	
+	-	+	93	7	0.2		
+	Mg	+	87	13	0.5		
+	Mn	+	61	39	7	2.0	0.8

[a] Reactions were performed with 0.2 M NaCl, 0.1 M MCl_2 and 10 mg Na^+-montmorillonite 22A for 5 days at 20°C as described in the experimental section.
[b] Im = 0.1 M imidazole-HCl buffer (pH 6.5).
[c] M = unreacted monomer.

The oligomerization of the bisphosphate **1** (0.0055 M) with EDAC as condensating agent was stimulated in the presence of Na^+-montmorillonite 22A. Addition of M(II)-ions enhanced the oligomerization reaction substantially. In the presence of the mineral the total yield of oligomers was increased from 2.2 to 9% with Mg(II) and from 1.8 to 13% with Mn(II) (Table 1). The production of longer oligomers was most effective with Mn(II) (compare van Vliet et al., 1994b) and oligomers with a chain length of five were observed (Figure 3), while in the absence of the mineral only dimer was formed. The reactions were almost free of by-products.



Scheme 1. Possible mechanism for the reaction of compound **1** with EDAC in 0.1 M imidazole-HCl (pH 6.5).

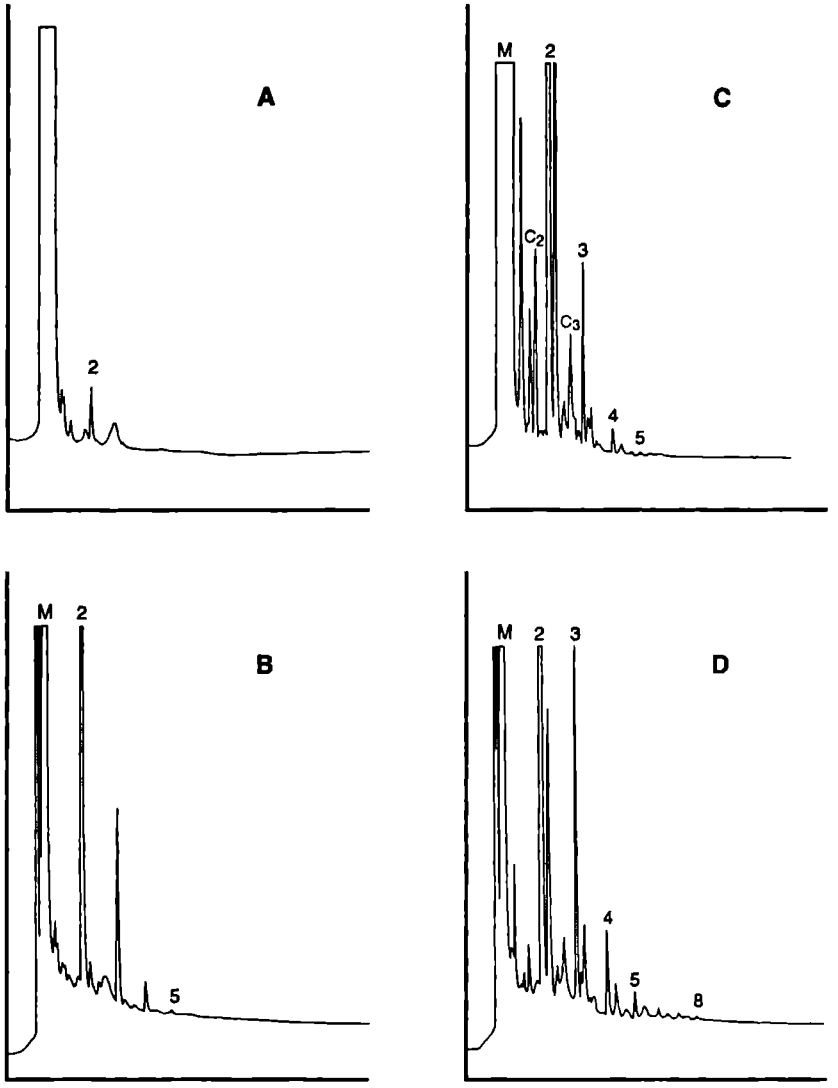


Figure 3. HPLC-chromatograms of the oligomerization products of compound **1** with FDAC A) Compound **1** alone, B) compound **1** in the presence of montmorillonite, C) compound **1** in 0 1 M imidazole-HCl (pH 6 5), D) compound **1** in 0 1 M imidazole-HCl (pH 6 5) in the presence of montmorillonite. Reactions were performed with 0 0055 M monomer **1**, 0 1 M $MnCl_2$, 0 2 M NaCl and 10 mg Na^+-montmorillonite 22A for 5 days at 20°C as described in the experimental section Analysis of RPC-5 in 0 02 M NaOH with a linear gradient of $NaClO_4$ (0-0 02 M over 30 min) at a flow rate of 1 0 ml/min UV absorption was monitored at 240 nm M=monomer, the numbers indicate the chain lengths of linear oligomers, C_2 and C_3 correspond with cyclic dimer and cyclic trimer, respectively

A higher extent of oligomerization was observed when the reactions were performed in an imidazole-HCl buffer (pH 6.5), although a substantial proportion of by-products was formed along with the oligomers (Figure 3). The oligomerization was stimulated by the presence of Na^+-montmorillonite (Table 1), but the proportion of by-products was only slightly decreased (Figure 3). Cyclic dimer and trimer were decreased substantial as is shown in Figure 3. Reaction in 0.1 M imidazole-HCl probably proceeds via a different mechanism than in the absence of this buffer. EDAC may react with a phosphate group to give a reactive intermediate as is illustrated in Scheme 1. This intermediate can react with a phosphate group to produce a pyrophosphate bond. In the presence of imidazole-HCl the EDAC group in this intermediate may be substituted by imidazole, which is present in a large excess, to generate a phosphoimidazolide group (Ivanovskaya et al., 1987; Visscher and Schwartz, 1988), which can react with a phosphate group to form a pyrophosphate linkage. The additional peaks observed in the HPLC-chromatograms of the reactions in imidazole-HCl (Figure 3) may be due to modification of the heterocyclic ring of the analogues by EDAC. The pH of mixtures with imidazole-HCl ($pH \approx 6.5$) was somewhat higher than those without imidazole-HCl ($pH \approx 6$). From the literature it is known that heterocyclic base modification by EDAC can take place easily at $pH > 6$, while at $pH \leq 6$ this modification is of less importance (Ivanovskaya et al., 1987). At higher pH keto-enol tautomerism might occur (pK=9.5, see Chapter 5), favoring a nucleophilic attack of the ring oxygen on the carbodiimide.

To study the effect of the concentration of the monomer we also conducted experiments with 0.01 M compound **1**. Table 2 shows that the results are comparable with the reactions under more dilute conditions. To improve the conversion of monomers into oligomers a second activation with EDAC was performed. Only an increase of the trimer and longer oligomers was observed. We next explored the influence of montmorillonite on the oligomerization by doubling the amount of clay mineral. From Table 2 it is observed that under most conditions the conversion of monomers to oligomers increased almost twice. The effect was higher on longer oligomers and in the absence of divalent metal ions even a trace of pentamer was produced, while in the presence of Mn(II) hexamers were formed (Table 2).

The above results are consistent with the hypothesis that oligomerization takes place on the mineral surface. A possible model is shown in Figure 4 (Kawamura and Ferris, 1994). The nucleotide analogues are concentrated on the mineral surface by interaction with their phosphate groups, facilitating condensation to form pyrophosphate linked oligomers. The oligomerization seems almost unaffected by an increased concentration of the monomer (compare Table 1 and 2), suggesting that all the binding sites on the mineral surface are covered with EDAC or products. Doubling the amount of mineral stimulated the extent of oligomerization almost twice.

Table 2. Oligomerization reactions of 0.01 M bisphosphate **1** with EDAC in the presence of Na^+-montmorillonite 22A.[a]

Mont (mg)	M(II)	EDAC[b]	M (%)[c]	Relative yield of oligomers of length *n* (%)				
				$n \geq 2$	$n \geq 3$	$n \geq 4$	$n \geq 5$	$n \geq 6$
-	-	1×	96	3.9				
-	Mg	1×	95	4.7				
-	Mn	1×	97	2.7				
10	-	1×	93	7	0.5			
10	Mg	1×	92	8	0.9	t[d]		
10	Mn	1×	86	14	1.4	0.2	t	
-	-	2×	96	3.7				
-	Mg	2×	95	5.4	0.2			
-	Mn	2×	96	4.4				
10	-	2×	92	8	1.3	t		
10	Mg	2×	91	9	1.6	0.1		
10	Mn	2×	87	13	3.0	0.4	t	
20	-	2×	87	13	2.3	0.2	t	
20	Mg	2×	84	16	3.3	0.4	0.1	
20	Mn	2×	79	21	5.1	1.0	0.4	t

[a] Reactions were performed with 0.2 M NaCl, 0.1 M MCl_2 and 10/20 mg Na^+-montmorillonite 22A for 7 days at 20°C as described in the experimental section.
[b] one addition of EDAC (1×) and after 3 days a second addition of EDAC (2×) of five equivalents per phosphate group as described in the experimental section.
[c] M = unreacted monomer.
[d] t = trace amounts of oligomers.

Oligomerization of compound **1a** *on Na^+-montmorillonite 22A.*

Oligomerization of the phosphoimidazolide **1a** was also catalyzed by the presence of Na^+-montmorillonite (Table 3). Without divalent metal ions the total yield of oligomers was increased from 4.7 to 9%, but the maximum length of the oligomers formed was unaffected by the clay mineral. Although the increase in the total yield of oligomers is not dramatic in the presence of divalent metal ions, the formation of longer oligomers is much more significant. In the presence of Mn(II) the yield of tetramers was increased from 0.2 to 3.0% and oligomers

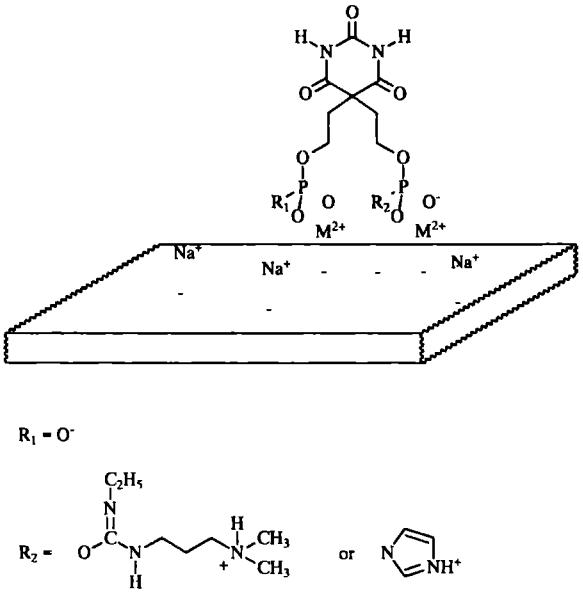


Figure 4. Proposed binding of compound **1** to the surface of Na^+-montmorillonite. $M^{2+} = Mg^{2+}$ or Mn^{2+}.

were produced with chain lengths up to 8. No cyclic products were observed, whereas in the absence of montmorillonite 3% cyclic dimer was formed. When the reactions of the bisphosphoimidazolide **1a** and the bisphosphate **1** with EDAC are compared, it is noticed that the global effect of montmorillonite seems comparable in both series of reactions. The more efficient oligomerization of **1a** was already suggested by the results of the oligomerization of compound **1** with EDAC in the presence of imidazole-HCl, since in that reaction a phosphoimidazolide group is also formed.

In contrast with the results of the reactions of the bisphosphate **1** with EDAC in 0.1 M imidazole-HCl, no by-products were observed if the reactions of the bisphosphoimidazolide **1a** were performed in this buffer. Although reactions of the bisphosphoimidazolides **1a** in 0.1 M imidazole-HCl are stimulated in the presence of montmorillonite, they showed a decreased yield of, especially, the longer oligomers if compared with reactions in the absence of imidazole-HCl (Table 3). Without the clay mineral both oligomerizations are comparable. These results are in agreement with those of Ferris and Ertem (1992b). An explanation could be that imidazole-HCl competes with inorganic cations for adsorption on the mineral surface, decreasing the amount of binding places for nucleotide analogues. These results are in line with the observation that oligomerization of 3',5'-bisphosphoimidazolides of guanosine was unaffected

by several minerals, clay minerals and polypeptides (Schwartz et al., 1987), since these reactions were performed in 0.4 M imidazole-HCl.

Table 3. Oligomerization reactions of 0.01 M bisphosphoimidazolide **1a** in the presence of Na^+-montmorillonite 22A.[a]

Mont	M(II)	Im[b]	M (%)[c]	Relative yield of oligomers of length *n* (%)					
				C2[d]	$n \geq 2$	$n \geq 3$	$n \geq 4$	$n \geq 5$	$n \geq 8$
-	-	-	95		4.7	1.0			
-	Mg	-	91		9	1.0			
-	Mn	-	71	3	26	7	0.2		
+	-	-	91		9	1.5			
+	Mg	-	85		15	2.5	0.4	0.2	
+	Mn	-	64		34	11	3.0	1.0	0.2
-	-	+	95		5.0	0.8			
-	Mg	+	93		7	0.5			
-	Mn	+	75	2	25	4.5			
+	-	+	90		10	1.0			
+	Mg	+	93		7	1.0			
+	Mn	+	70		30	6	1.2	0.4	

[a] Reactions were performed with 0.1 M NaCl, 0.2 M MCl_2 with 20 mg Na^+-montmorillonite 22A for 7 days at 20°C.
[b] Im = 0.1 M imidazole-HCl buffer (pH 6.5).
[c] M = unreacted monomer.
[d] C2 = cyclic dimer.

Acknowledgements.
We thank J.P. Ferris for a gift of Montmorillonite 22A and A.H. Hill for a gift of RPC-5.

Oligomerization Study of a Set of Six Related Achiral Nucleotide Analogues in Aqueous Solution. Influence of the Nature of the Heterocyclic Ring on the Reaction.

Abstract

The oligomerization reactions of six achiral nucleotide analogues - 5,5-di(2-phosphoethyl) pyrimidines related to barbituric acid - are described in aqueous solution at various pH values. The ionization constants of the heterocyclic rings of these analogues have been determined. After activation the monomers were oligomerized, producing pyrophosphate-linked chains in high yield. An exception was the behavior of analogue **2a**, in which an intramolecular reaction of the phosphoimidazolide groups with the exocyclic oxygen atoms of the heterocyclic ring resulted in the formation of cyclic compound **26**. Oligomerization of compound **3a** yielded branched oligomers, due to an intermolecular reaction of the phosphoimidazolide groups with the ring. Keto-enol tautomerism in the pyrimidine bases seems responsible for these results. The limiting factor for the production of high molecular weight oligomers is probably the formation of cyclic oligomers. It is proposed that the production of these cyclic oligomers is inversely correlated to the degree of complexation of the phosphate-coordinated metal ions to the ring. Some possible prebiotic implications of these results are discussed.

Introduction

In the literature many oligomerization studies with modified nucleotides have been reported. In some investigations the (deoxy)ribose residue in the nucleotides was replaced by a flexible, acyclic sugar alcohol (Tohidi and Orgel, 1989; Visscher and Schwartz, 1989; Stribling and Miller, 1991). Reactions have also been described in which a pyrophosphate, phosphoramide, amide or carbonyl ester linkage is formed rather than a phosphodiester (Schwartz and Orgel, 1985a; Zielinski and Orgel, 1985; Cheikh and Orgel, 1990; Harada and Orgel, 1990). In only a few studies has the base been replaced by another heterocyclic residue, usually a related purine. For example, reactions were performed with activated nucleotide analogues of 2,4-diaminopurine, hypoxanthine, 7-deazaguanine and N3-bonded purines (N3-adenine, N3-xanthine and N3-isoguanine) (Webb and Orgel, 1982; Schwartz et al., 1987; Rembold et al., 1994; Hill et al., 1988; Hill et al., 1991). No analogues with modified pyrimidine rings were used, presumably because of the poor stacking interactions of pyrimidines. It is well known that purine bases have higher stacking interactions and as a result oligomerization reactions of purine analogues are more efficient than those of pyrimidine analogues (Visscher and Schwartz, 1990a). In spite of favorable stacking interactions, however, the oligomerization of an activated bisphosphate of deoxyinosine was found to be unexpectedly inefficient and a substantial amount of side products was formed (Schwartz et al., 1987). This suggests that other factors also play a part in determining the course of oligomerizations.

In our search for potential precursors of RNA molecules we introduced a new set of nucleotide analogues in which the sugar group as well as the heterocyclic base was altered (Schwartz, 1993). Reaction of these activated analogues gives pyrophosphate-linked oligomers. Earlier results from studies of compound **1** showed a large extent of oligomerization in aqueous solution (van Vliet et al, 1994b). Recently, we synthesized the complete set of analogues (van Vliet et al, 1994a) and describe here oligomerization studies of all the compounds at various pH values. The oligomerization behavior of the analogues can be related to the pK as well as to the number of amino groups in the heterocyclic ring, which in turn is correlated with the number of nonprotonated endocyclic N-atoms.

Experimental

Material and methods.

Ultraviolet spectra were recorded with a Beckman DU-40 spectrophotometer using cells of 1.0 cm pathlength. Measurements of pH were made using a Knick pH-meter 761 calimatic

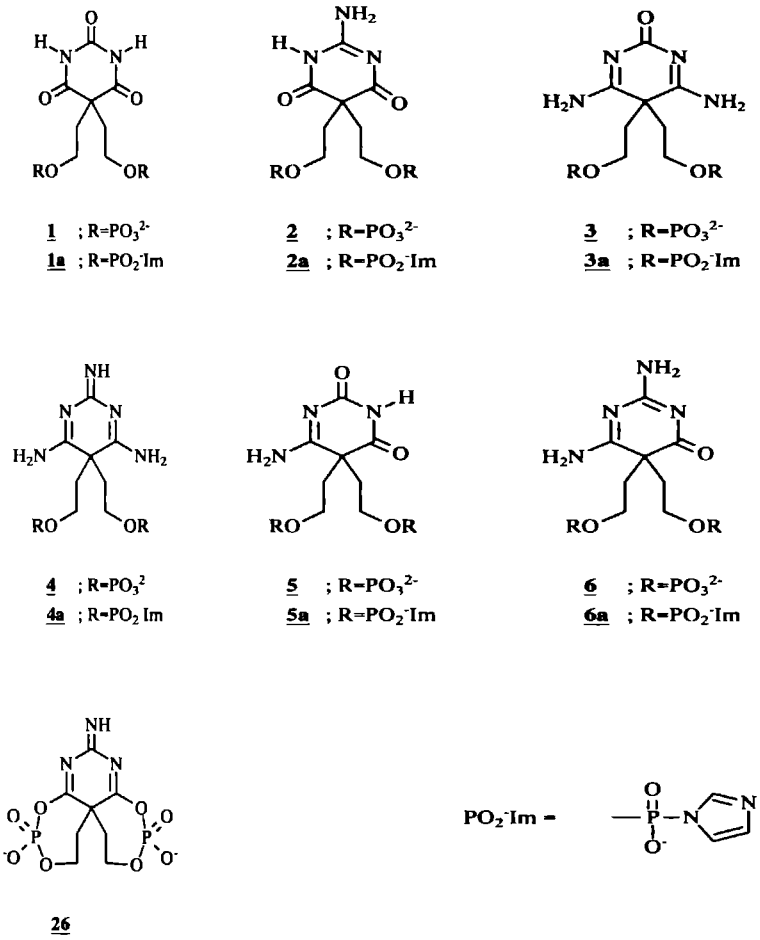


Figure 1.

with a Russel pH-electrode type RUCMAWL at room temperature. Standards at pH 3.00, 8.00, 10.00 and 13.00 were obtained from Merck. Nuclear Magnetic Resonance spectra were recorded with a Bruker 400 MHz spectrometer. HPLC analysis of oligomers was carried out on an RPC-5 column in 0.02 M NaOH with a linear gradient of $NaClO_4$ (0.03 M over 45 min for reaction products of **1**, **2**, **3**, **4** and **5** and 0.018 M over 45 min for reaction products of **6**) at 1.0 ml/min. The eluent was monitored at 240, 250, 260, 265, 270 or 280 nm. Pyrophosphatase (type II from *Crotalus ademateus* venom) and alkaline phosphatase (type III from *Escherichia coli*) were purchased from Sigma. The compounds **1-6** and **1a** were synthesized as described earlier (van Vliet et al., 1994a; van Vliet et al., 1994b; Chapters 2 and 3).

Determination of the ionization constants (pK's).

The ionization constants of the analogues **1-6** were determined by absorbance spectrophotometry. UV absorption of the compounds **1** to **6** were measured over a pH range from 2 to 13 at room temperature and are illustrated in Figure 2. The solutions of various pH values were prepared by dilution of a concentrated stock solution of each compound with an appropriate buffer. After the absorbance spectrum was recorded, the pH of each solution was measured. The following buffers were used at a concentration of 0.05 M: KCl-HCl (pH 1.0-2.2), Warpole's acetate (pH 3.0-6.0), KH_2PO_4-HCl (pH 6.0-8.0), Tris-HCl (pH 7.0-9.0), Borax-HCl (pH 8.0-10.0), $NaHCO_3$-NaOH (pH 9.5-11.0), Na_2HPO_4-NaOH (pH 11.0-12.0) and KCl-NaOH (pH 12.0-13.0).

General procedure for the synthesis of compounds **2a-5a**.

The triethylammonium salt of each of the compounds **2-5** (400 μmol) was converted into a tributylammonium salt on Dowex A6 50W-X8 (tributylammonium form) using a mixture of water and ethanol. The eluent was evaporated to dryness, coevaporated with ethanol and the residue was dried under vacuum. The tributylammonium salt was dissolved in DMSO (2.0 ml) and added dropwise to an anhydrous solution of imidazole (2.72 g, 40 mmol), triphenylphosphine (539 mg, 2.0 mmol) and 2,2'-dithiodipyridine (441 mg, 2.0 mmol) in DMF (4.0 ml), which was stirred for 2 h under argon at room temperature. The product was precipitated by addition of an anhydrous mixture of acetone (50 ml), diethylether (50 ml), TEA (4 ml) and a saturated solution of $NaClO_4$ in acetone (0.5 ml). The suspension was centrifuged, the supernatant was removed and the residue was washed three times with an anhydrous mixture of acetone-diethylether (1:1) and finally twice with anhydrous diethylether. After drying under vacuum, each of the products was obtained as a white powder. Yields: 83% (**2a**), 91% (**3a**), 78% (**4a**), 95% (**5a**), and 75% (**6a**). The identity of the products was confirmed by UV and by HPLC after hydrolysis with sodium acetate.

Compound **26**.

Compound **26** was synthesized from analogue **2** as described above - for compounds **2a-6a** - with 4 instead of 100 equivalents of imidazole.

Yield: 72%. ^{31}P-NMR (d_6-DMSO, external trimethylphosphate) δ ppm: -6.472 (s). ^{1}H-NMR (d_6-DMSO, external TMS) δ ppm: 2.549 (s (br), 4H, 2×CH_2), 3.850 (s (br), 4H, 2×CH_2O), 6.654 (s (br), OH), 11.362 (s (br), NH). ^{31}P-NMR (D_2O, external trimethylphosphate) δ ppm: -6.885 (s). ^{1}H-NMR (D_2O, external TMS) δ ppm: 2.728-2.753 (t, 4H, 2×CH_2, J=5.05 Hz), 4.065-4.129 (q, 4H, 2×CH_2O, J=5.05 Hz). UV (H_2O) λ (nm) (ϵ): λ_{max} = 230 (8100), 267.5 (7200) in 0.1 M NaOH.

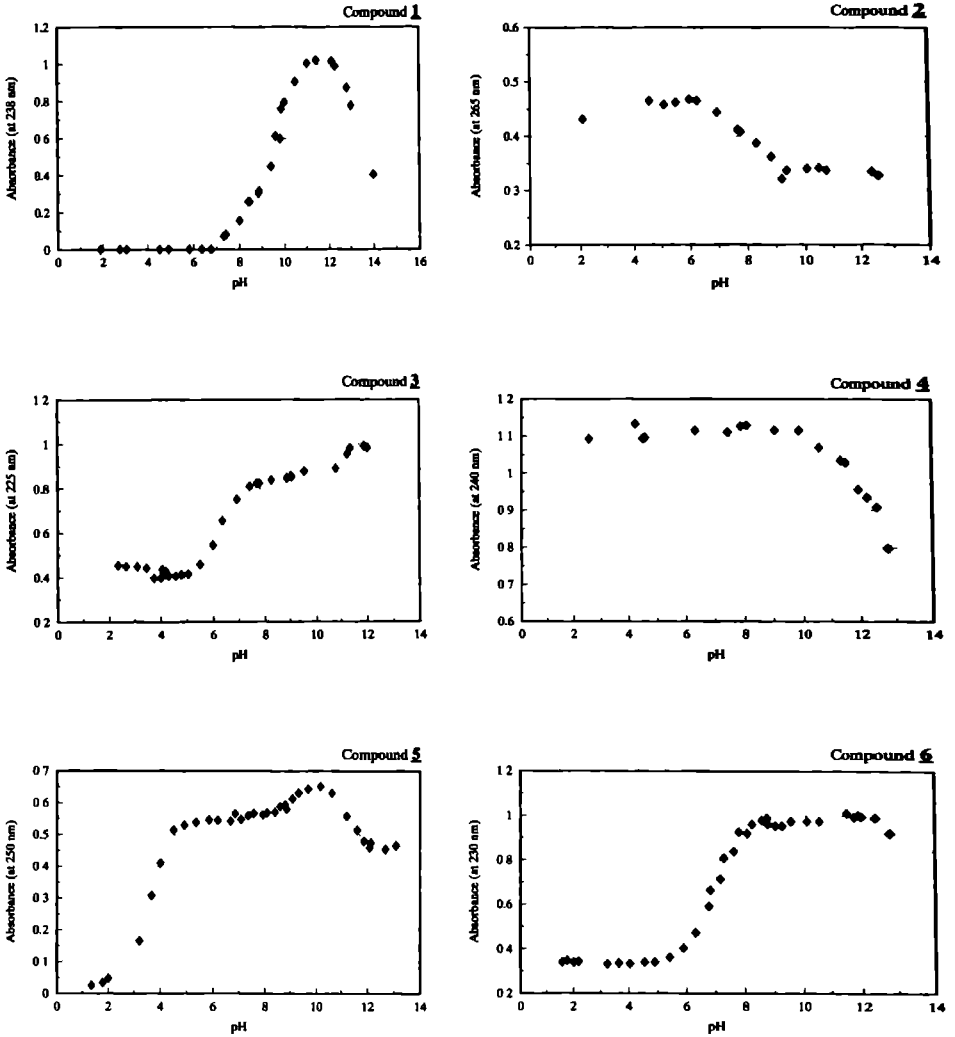


Figure 2. UV-absorption of the compounds **1-6** as a function of pH.

General treatment of compounds **1a-6a** *with sodium acetate.*

The compounds (0.05 μmol) **1a-6a** were incubated with sodium acetate (0.1 M, pH 4.0) at 50°C. Samples were taken after 5, 15 and 60 minutes and analyzed by HPLC on a C18 reverse phase column. The imidazole groups were hydrolyzed sequentially, producing compounds with one and ultimately two free phosphate groups (**1-6**).

Oligomerization reactions in aqueous solutions.

All reactions were performed in Eppendorf tubes in a total volume of 10 μl. First, $MgCl_2$ (4 μl, 1.0 M) or $MnCl_2$ (4 μl, 1.0 M) and NaCl (1 μl, 1.0 M) were added to each tube, followed by concentration of the mixtures to dryness. The residues were redissolved in an appropriate buffer (4 μl, 1.0 M) and a freshly prepared solution of activated monomer (6 μl, 0.083 M) was added at 0°C. Finally, the reaction mixtures were mixed, centrifuged and incubated for 4 days at 37°C. The reactions were quenched by addition of KEDTA (8 μl, 1.0 M), diluted with water to a volume of 100 μl and stored at -25°C. For HPLC-analysis aliquots with a volume of 10 μl were taken from the mixtures. Surviving imidazolides were hydrolyzed by incubation in a sodium acetate buffer (pH 4.0, 0.1 M, 100 μl) for 1 h at 50°C.

The reactions were performed with the following buffers: 2,6-lutidine-HCl (pH 5.0) and imidazole-HCl (pH 6.5 and pH 8.0).

Identification of isolated oligomers.

Reaction products were fractionated by HPLC on an RPC-5 column and neutralized with HCl (6 M). The purity of the isolated oligomers was determined by reinjection of the fractions on an RPC-5 column. Identification of isolated oligomers was by enzymatic digestions and chemical hydrolysis.

Enzyme digestions and chemical hydrolysis.

Pyrophosphatase digestions were performed on isolated oligomers (0.05 ODU) in a Tris-HCl buffer (0.1 M, 100 μl, pH 7.2) containing $MgCl_2$ (0.04 M) with 0.2 units enzyme. Incubation was for 5 h at 37°C. Alkaline phosphatase treatment was performed on samples in a Tris-HCl buffer (0.04 M, 100 μl, pH 8.0) containing $MgCl_2$ (0.02 M) with 0.2 units enzyme for 4 h at 37°C. After incubation KEDTA (4 μl, 1.0 M, pH 9.0) was added and the mixtures were analyzed by HPLC.

Chemical hydrolysis of the pyrophosphate linkages was performed by treatment with $ZrCl_4$. To samples of isolated oligomers (75 μl, 0.05 ODU) a solution of sodium acetate (13 μl, 3.0 M, pH 5.0) and $ZrCl_4$ (3 μl, 1.0 M) were added and the mixtures were incubated for various times at 50°C. The reactions were quenched by addition of KEDTA (9 μl, 1.0 M, pH 9.0) and

neutralized with NaOH (6.7 μl, 10 M). After filtration the mixtures were analyzed by HPLC.

Molecular modeling.

Molecular mechanics studies were performed using the AMBER force field from the Hyperchem program (Autodesk, Inc 1993, 2320 Marinship Way, Sausalito, CA 94965, USA). No solvent molecules were included.

Results

Ionization constants.

The ionization constants (pK-values) of the rings of the analogues were determined by absorbance spectrophotometry for the pH range 2 to 13. The results are presented in Table 1. Endocyclic nitrogens are rather basic in character than exocyclic amine nitrogens and as a result hydrogens at endocylic N-atoms are removed more easily at alkaline pH (Saenger, 1984). Conversely, since amide-like hydrogens are more acidic in character than amine-like hydrogens deprotonation of endocyclic nitrogens rather occurs than deprotonation of exocyclic amine nitrogens. Because of the reasons mentioned, we ascribe the ionization constants to the endocyclic N-atoms.

Table 1. Ionization constants (pK-values) of the heterocyclic ring of achiral nucleotide analogues **1-6**.[a]

Compound	pK	
1		9.5, >12
2	<2[b],	7.9
3	<2, 6.1	
4	<2, <2,	>11
5	3.6,	9.2, 11.3
6	<2, 6.9	

[a]the pK-values were determined by absorbance spectrophotometry as described in the experimental section.
[b]undetectable in the pH range 2-13.

Imidazolation reactions.

Imidazolation of the analogues **1-6** (Figure 1) was achieved by a method described earlier (Van Vliet et al., 1994b). Because of solubility problems the triethylammonium salts of com-

pounds **2-6** were converted into their tributylammonium salts. Reaction of compound **2** in the presence of 4 equivalents of imidazole yielded a main product with a changed UV-spectrum, indicating that the heterocyclic ring of the product was modified. Treatment of this product with 80% acetic acid at 50°C resulted, via an intermediate, in recovery of starting compound **2** quantitatively. The product does not contain imidazole groups, as was shown by the ^{1}H-NMR spectrum. A ^{31}P-NMR spectrum of the compound showed only one signal at 6.47 ppm, so the reaction product possesses two identical phosphate groups. The product was resistant to digestion with alkaline phosphatase and therefore contained no terminal phosphate groups. It was concluded that during the imidazolation reaction both phosphate groups were condensed with the ring, resulting in the formation of a tricyclic product. Molecular models and computational modeling confirmed this possibility. The structure of this product is illustrated as **26** in Figure 1. When the amount of imidazole was increased from 4 to 100 equivalents during the imidazolation of analogue **2**, compound **2a** was obtained (in 83% yield), as was shown by ^{31}P-NMR and ^{1}H-NMR. The imidazolides **3a-6a** were also obtained by this modification.

Table 2. Product distributions in the oligomerizations of the analogues **1a**, **3a-6a** at pH 6.5.

			Monomer (%)	Relative yield of oligomers of length *n* (%)				
Comp	Metal	Ctot[1]		*n*≥2	*n*≥5	*n*≥10	*n*>15	*n*≥20
1a	Mg	9	42	58	42	t[2]		
5a	Mg	6	33	67	11	0.4		
6a	Mg	4.4	26	74	19	1.3		
4a	Mg		28	72	15	0.4		
3a	Mg		23	77	63	27	11	4.6
1a	Mn	25	14	86	26	3.3	0.3	
5a	Mn	16	19	81	30	6	1.2	
6a	Mn	10	13	87	42	11	3.4	1.1
4a	Mn		13	87	38	8	1.3	t
3a	Mn		14	86	75	47	29	19

Conditions: 0.05 M monomer; 0.4 M MCl_2; 0.1 M NaCl; 0.4 M imidazole-HCl buffer; 4 days at 37 °C.
[1] Ctot is the total extent of cyclic pyrophosphate linked oligomers.
[2] t is trace amount of oligomers.

Oligomerization reactions.

Oligomerization studies of the analogues **1a-6a** were performed at a concentration of 0.05 M activated monomer in the presence of 0.4 M $MgCl_2$ or $MnCl_2$ and 0.1 M NaCl at pH 6.5 and 37°C. The reactions with the monomers **1a**, **3a-6a** produced pyrophosphate-linked oligomers in high yield. The longest oligomers as well as the highest conversion of the monomers to oligomers were produced in the presence of $MnCl_2$. HPLC-chromatograms of the reactions with Mn^{2+}-ions are shown in Figure 3. A large difference in the production of higher molecular weight oligomers was found (Tables 2-4). The formation of longer oligomers was most effective in the reactions of compound **3a**. In the presence of $MnCl_2$ oligomers with a chain length of 20 and longer were produced in a yield of 19% (Table 2). Another aspect of the reactions of compound **3a** was the formation of by-products. Especially in the presence of $MgCl_2$, a substantial proportion of these products was formed, as was concluded from the complex pattern of peaks in the chromatogram (not shown). In the presence of $MnCl_2$ the proportion of by-products was less substantial.

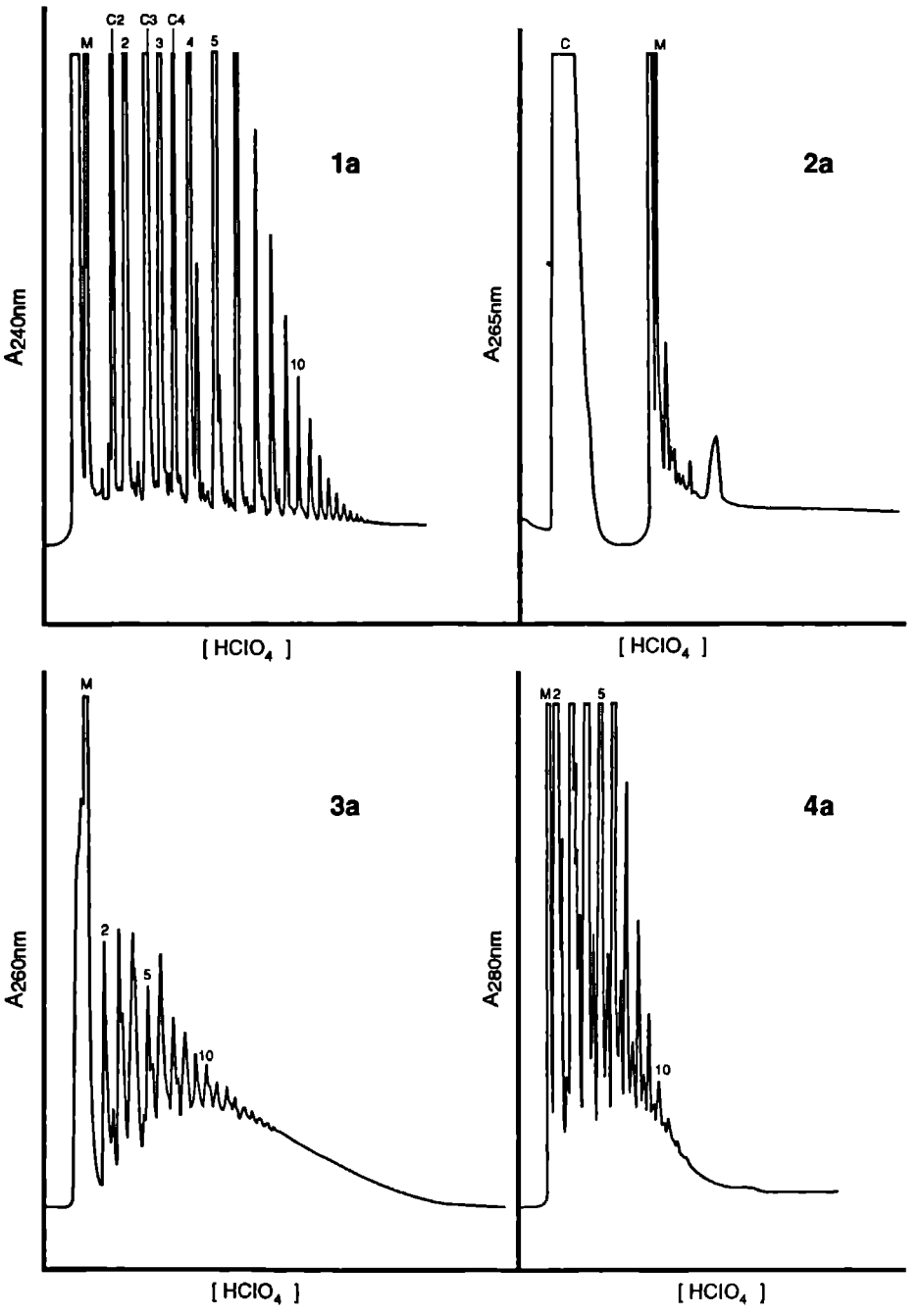
Table 3. Product distributions in the oligomerizations of the analogues **1a**, **3a-6a** at pH 5.0.

			Monomer (%)	Relative yield of oligomers of length *n* (%)				
Comp	Metal	Ctot[1]		$n \geq 2$	$n \geq 5$	$n > 10$	$n \geq 15$	$n \geq 20$
1a	Mg	4.4	52	48	2.5	t[2]		
5a	Mg	3.4	52	48	3.2			
6a	Mg	4.3	31	69	17	1.2		
4a	Mg		43	57	7	t		
3a	Mg		29	71	60	27	13	6.3
1a	Mn	25	15	85	28	4.2	0.5	t
5a	Mn	18	20	80	34	12	4.8	2.1
6a	Mn	11	11	89	52	27	15	8
4a	Mn		19	81	34	10	3.4	1.1
3a	Mn		15	85	79	59	46	36

Conditions: 0.05 M monomer; 0.4 M MCl_2; 0.1 M NaCl; 0.4 M imidazole-HCl buffer; 4 days at 37 °C.

[1] Ctot is the total extent of cyclic pyrophosphate linked oligomers.

[2] t is trace amount of oligomers.


M
C2
2
C3
3
C4
4
5
10
1a
A240nm
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C
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2a
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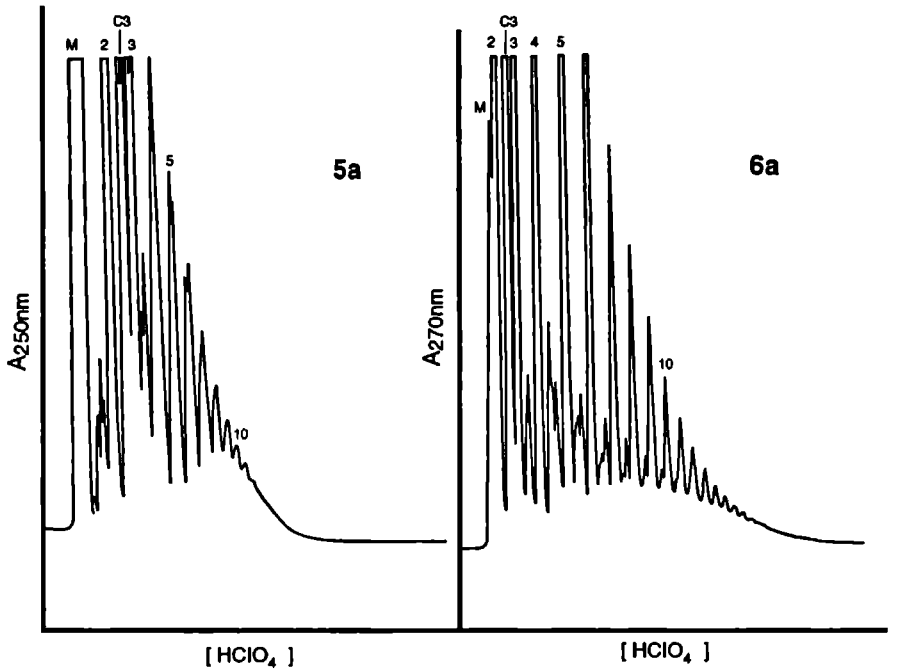


Figure 3. HPLC-chromatograms of the products of the oligomerization reactions of the analogues **1a-6a**. Conditions: 0.05 M activated monomer, 0.4 M $MnCl_2$, 0.1 M NaCl, and 0.4 M imidazole-HCl buffer (pH 6.5) for 4 days at 37°C. M=monomer, C=tricyclic compound **26**. The numbers above the peaks are the chain lengths of the corresponding linear oligomers. The peaks labelled with C2, C3 and C4 correspond to pyrophosphate-linked cyclic dimer, trimer and tetramer, respectively. (For HPLC conditions see experimental).

The chromatogram of compound **5a** also shows a complex pattern of peaks. We believe that, in this case, every cluster of peaks consists of stereoisomers with the same chain length. Compound **5** is a prochiral molecule and incorporation of each monomer into a growing chain will create a new chiral center. Consequently, oligomers can have many configurations. The distance of the phosphate groups in the pyrophosphate backbone to the endocyclic N-atoms will depend on the overall configuration of the chain. The actual pK-value of residues of compound **5** in an oligomer (measured as 11.3 for the monomer) is dependent on the distance of a phosphate group to the endocyclic N-atom (Saenger, 1984) and probably also on the proximity of an exocyclic =O or NH_2 function. As a result, at pH 12 the degree of deprotonation of the endocyclic N-atoms will be dependent on the configuration of the oligomers. Separation of the oligomers on an RPC-5 column is based, among other factors, on

the total negative charge. At pH 12, therefore, differences in configuration of the oligomers can result in small differences in retention times on HPLC. Compound **6** is also a prochiral molecule and, as a result, oligomers of this analogue can also assume multiple configurations. In contrast to compound **5**, this analogue does not have an ionization constant in the pH range 7 to 13. Because of this the total negative charge at pH 12 will be the same for all configurations of oligomers with the same chain length, which probably explains the relatively simple HPLC pattern of **6a**.

An exception in the series of oligomerization experiments was the reaction of analogue **2a**, which produced hardly any oligomerization products (Figure 3). The main product of this reaction was identified as the tricyclic compound **26**.

In addition to the special case of tricyclic monomer formation noted for analogue **2a**, cyclic pyrophosphate-linked oligomers were observed among the oligomerization products. The degree of cyclization appears to be inversely correlated with the number of exocyclic amino groups for compounds **1a**, **4a**, **5a** , **6a**. (see Figure 4). The formation of these products will be discussed below. To investigate the influence of the pH on the oligomerization, reactions were performed at pH 5.0, 6.5 and 8.0. The results are presented in the Tables 2-4. In the presence of $MgCl_2$ the oligomerizations were more efficient at pH 6.5 than at lower or higher pH, except for the reaction of component **3a**, which was more efficient at pH 5.0. The reactions showed a different tendency in the presence of $MnCl_2$; the efficiency of the reactions increased with decreasing pH. At pH 8.0 the efficiency of the reactions decreased in the order: **3a** > **4a** > **6a** > **5a** > **1a**. At pH 5.0 and 6.5 the extent of oligomerization with analogue **6a** was higher than with analogue **4a**.

Characterization of the oligomerization products.

The products of the oligomerization reactions were isolated from an RPC-5 column and characterized by enzymatic and chemical hydrolysis. Isolated oligomers were degraded by $ZrCl_4$ (Visscher and Schwartz, 1992) and by pyrophosphatase to establish the length of the oligomers. For example, a pentamer was hydrolyzed with $ZrCl_4$ producing the tetramer, trimer, dimer and monomer in increasing proportions with time. Similar results were achieved by digestion with pyrophosphatase. Terminal phosphate groups of isolated oligomers were removed by treatment with alkaline phosphatase, which resulted in the formation of a single peak in the HPLC chromatogram with a smaller retention time than the parent compound. An exception was the digestion of oligomers of compound **3a** with alkaline phosphatase. For example, treatment of an isolated fraction corresponding to an heptamer of **3**, produced several peaks with smaller retention times. Cyclic products were resistant to the action of alkaline phosphatase and were degraded via the corresponding linear oligomers in the presence

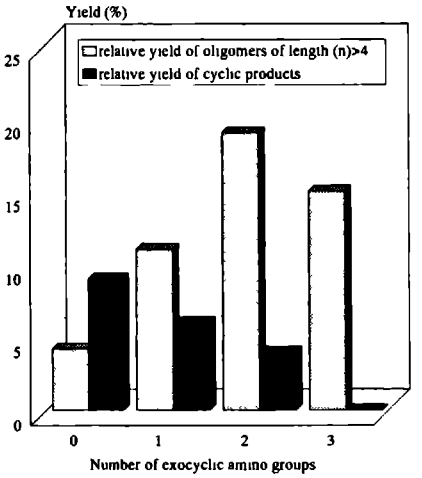


Figure 4. Relation of the number of exocyclic amino groups with the product distribution of the oligomerization of the analogues **1a**, **4a-6a**. Conditions: 0.05 M activated monomer, 0.4 M $MgCl_2$, 0.1 M NaCl, and 0.4 M imidazole-HCl buffer (pH 6.5) for 4 days at 37°C.

Table 4. Product distributions in the oligomerizations of the analogues **1a**, **3a-6a** at pH 8.0.

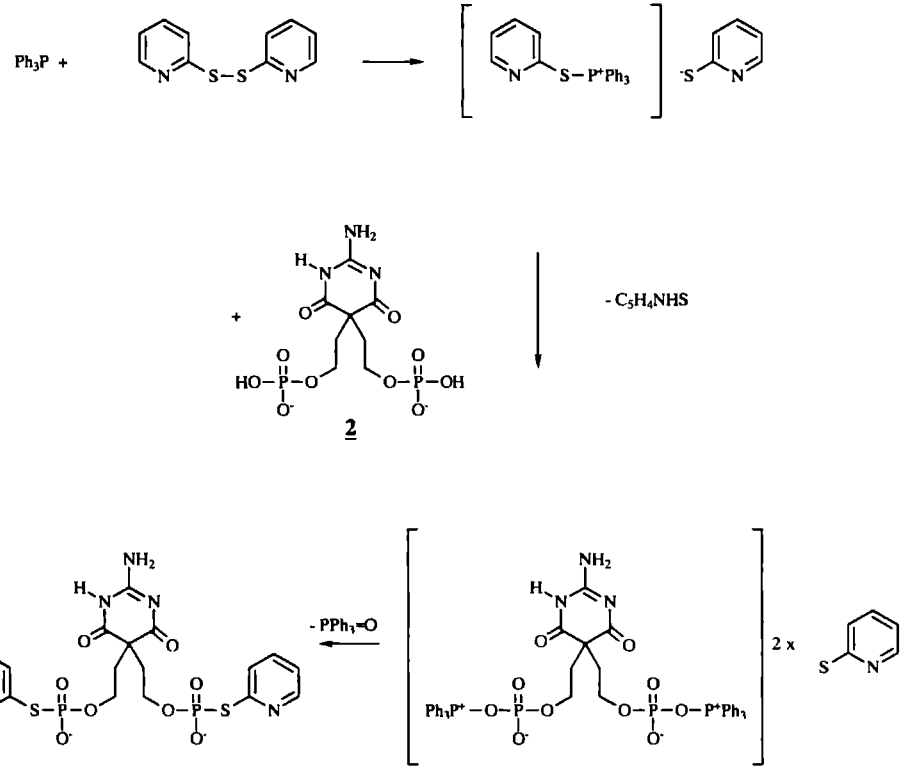
Comp	Metal	Ctot[1]	Monomer (%)	Relative yield of oligomers of length *n* (%)				
				n>2	*n*≥5	*n*≥10	*n*≥15	*n*>20
1a	Mg	2.1	70	30	0.3			
5a	Mg	2.0	55	45	1.9			
6a	Mg		47	53	4.6			
4a	Mg		46	54	5.4			
3a	Mg		48	52	30	5.1	0.6	
1a	Mn	4.4	45	55	3.6	t[2]		
5a	Mn	2.4	44	56	5.6	t		
6a	Mn	2.5	46	54	7	0.7		
4a	Mn		38	62	10	0.2		
3a	Mn		42	58	34	8	2.2	0.7

Conditions: 0.05 M monomer; 0.4 M MCl_2; 0.1 M NaCl; 0.4 M imidazole-HCl buffer; 4 days at 37 °C.
[1] Ctot is the total extent of cyclic pyrophosphate linked oligomers.
[2] t is trace amount of oligomers.

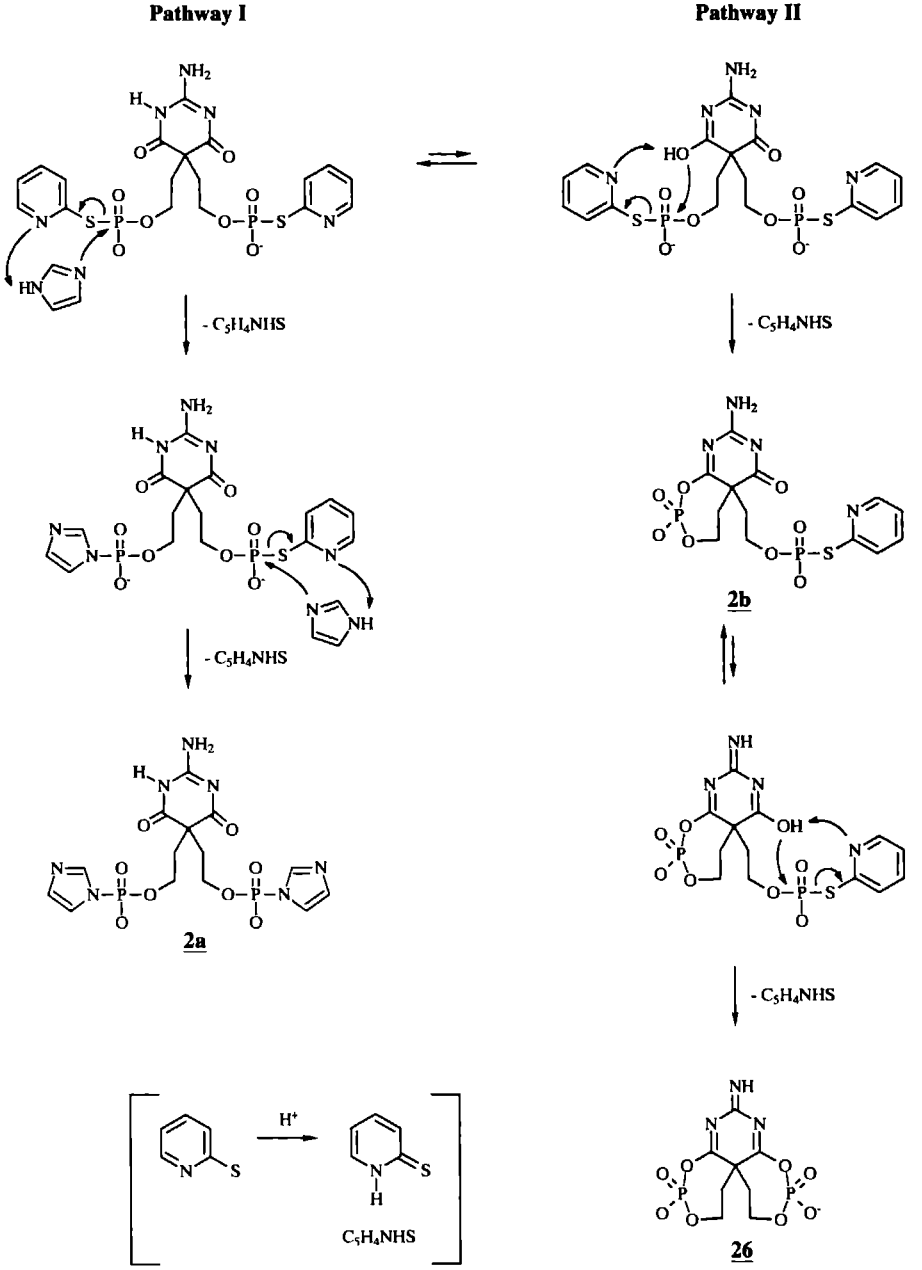
of $ZrCl_4$ or pyrophosphatase.

Discussion

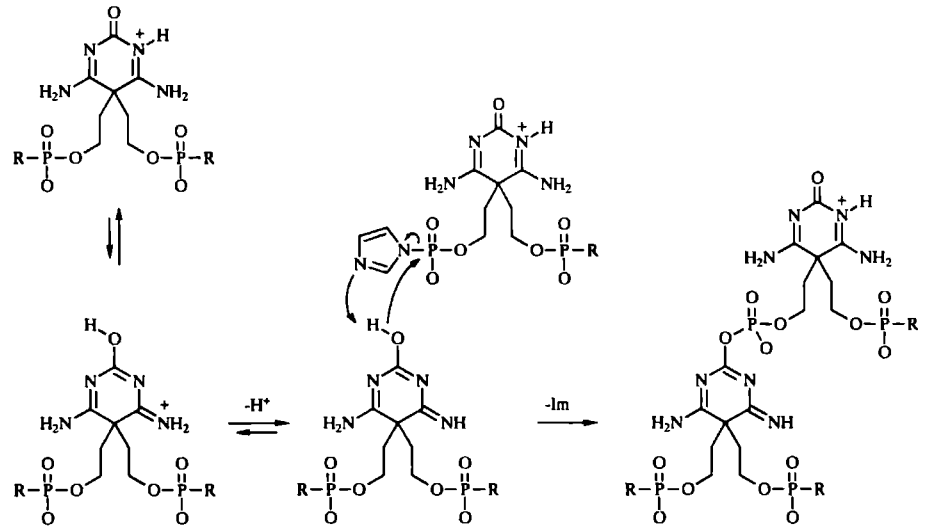
The oligomerization of the barbituric acid derivatives **1a-6a** is dependent on the nature of the pyrimidine ring. Reaction of compound **2a** gave almost no oligomerization products under the conditions employed. An explanation could be a rapid equilibration between the keto- and the enol-form of compound **2a** (Elguero et al., 1976; Beak, 1977) leading to cyclization. The endocyclic nitrogen atom of analogue **2** (pK=7.9) has the tendency to donate a proton, which



Scheme 1. Possible mechanism for the imidazolation of analogue **2**. When 4 equivalents of imidazole are used, the reaction will proceed via pathway **II** and compound **26** is obtained. When a large excess (100 equivalents) of imidazole is used the reaction will be driven in the direction of pathway I and compound **2a** is obtained. The driving force in these reactions is the isomerization of pyridine-2-thiol to the pyridine-2-thione tautomer (insert), which is thermodynamically more stable and unreactive with respect to the other components (Mukaiyama et al., 1970).


Pathway I
Pathway II
- C5H4NHS
- C5H4NHS
2b
- C5H4NHS
2a
- C5H4NHS
H+
C5H4NHS
26

will be facilitated by the proximity of the negative charge of a phosphate group (Saenger, 1984). Because of this migration, a tautomeric change from the keto- to the enol-form would take place easily. As a result, the exocyclic oxygen atoms of the enol-form could react with the phosphoimidazolide groups to give compound **26** as the main product. A plausible pathway for the imidazolation of compound **2** and the formation of the cyclic product **26** is given in Scheme 1. First, a thiophosphodiester of analogue **2** is formed (Nakagawa et al., 1983). This phosphodiester of pyridine-2-thiol is particularly reactive since there is an additional driving force in the formation of the more stable pyridine-2-thione tautomer (Mukaiyama et al., 1970). Then, tautomerisation of the keto-form into the enol-form might take place (Elguero et al., 1976). In this isomer a nucleophilic attack of the exocyclic oxygen atom on the phosphorus atom would result in the formation of intermediate **2b**. After a second enolization, compound **26** would be formed as is shown in Scheme 1. When an excess of imidazole is used, a nucleophilic attack of an imidazole molecule - instead of the exocylic oxygen atom - on the phosphorus atom is more likely. Therefore, the reaction would be driven in the direction of pathway I and compound **2a** would be obtained.



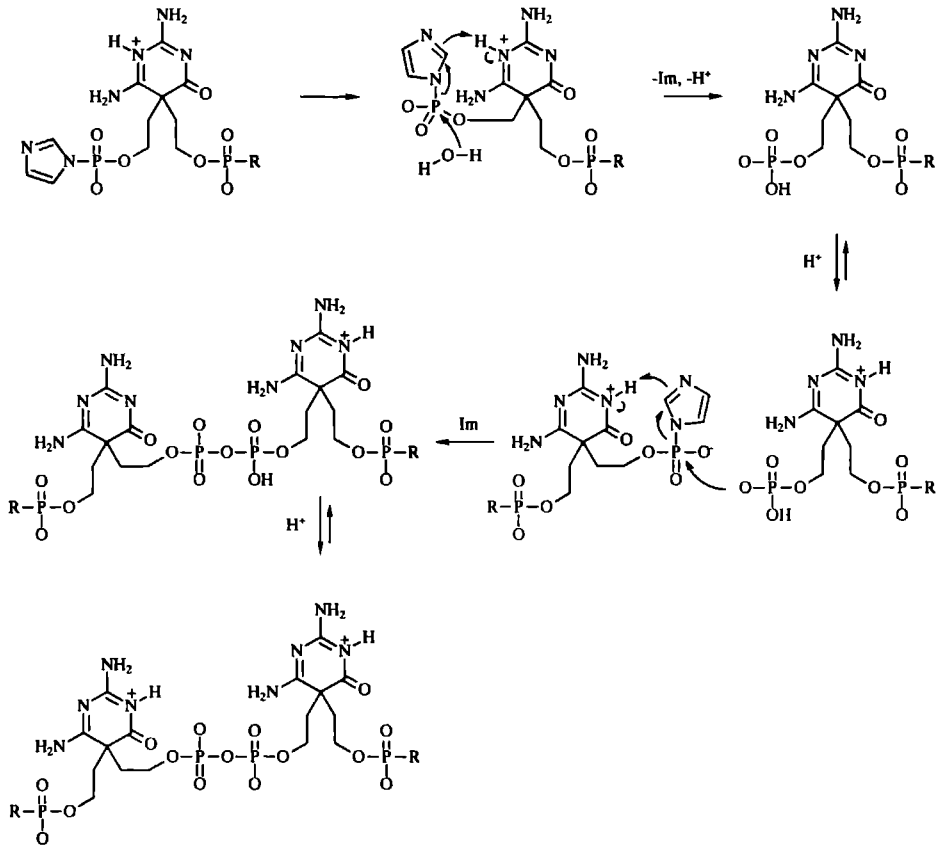
Scheme 2. Possible mechanism of the formation of branched products in the oligomerization of analogue **3a**.

At pH 8.0 the oligomerization reaction is more efficient when the pyrimidine ring contains more amino groups (see Tables 2-4). The exception to this observation is the reaction of compound **3a**, which produced the highest yield of long oligomers, while the pyrimidine

ring of this compound contains only one amino group. The large quantity of side products formed with this compound is also remarkable. A reason could be a keto-enol tautomerism after protonation of the endocyclic nitrogen atom (pK=6.1). In the enol-isomer the exocyclic oxygen atom can react with a phosphoimidazolide group to yield a branched product with three instead of two terminal phosphate groups (see Scheme 2). In this manner large branched polymers could be formed. This assumption is supported by enzymatic degradation. Alkaline phosphatase treatment of an isolated fraction, corresponding to a heptamer, produced more than one peak, indicating that a variable number of terminal phosphate groups were cleaved. Reactions with $MnCl_2$ resulted in a smaller proportion of branched products than with $MgCl_2$. Since Mn(II) specifically catalyzes the formation of pyrophosphate bonds, this effect would be expected to result in a relative decrease in the proportion of phosphodiester chain branches.

At pH 5.0 and 6.5 the extent of oligomerization of analogue **6a** was higher than that of analogue **4a**. For an explanation we have to look at the proposed mechanism of the reaction which is given in Scheme 3. First, a proportion of the phosphoimidazolide groups are hydrolyzed to free phosphates, which is facilitated by protonation of the leaving group. Then the pyrophosphate bonds are formed via a nucleophilic attack by the terminal phosphate groups at the phosphoimidazolide groups, catalyzed by a divalent metal ion (Schwartz and Orgel, 1985a). This reaction may also be assisted by protonation of the imidazole groups. At neutral pH protonation of the imidazole groups is mainly caused by water (Kanavarioti et al., 1989). However, at pH 6.5 and 5.0 it could also be accomplished by an intramolecular attack of the protonated endocyclic nitrogen atoms of analogue **6a** (pK=6.9), which would result in an acceleration of the hydrolysis and condensation reaction (Joyce and Orgel, 1993).

If we consider the oligomerization yields of analogues **1a**, **5a** and **6a** (Figure 4), it is noticed that the formation of cyclic oligomers is inversely correlated with the number of exocylic amino groups. This in turn is correlated with the number of nonprotonated endocyclic N-atoms. An explanation for this relationship can be found in the complexation of metal ions with the ring. From molecular modeling studies it is suggested that, in a conformation in which the phosphate-coordinated metal ions are complexed with the heterocyclic ring, only formation of linear products is possible (Figure 5). Endocyclic N-atoms are the preferred binding sites for metal ion coordination in nucleic acid bases. In purine nucleotides it is found that Mn^{2+} ions are coordinated directly to the phosphate group and N-7 of the ring. Mg^{2+} ions are coordinated directly with the phosphate group and, via a water molecule, with N-7 of the ring (Pezzano and Podo, 1980; Sigel et al., 1987; Sigel et al., 1994). In addition, the degree of complexation with the ring, macrochelate formation, is dependent on the basicity of N-7 of the purine. For example, the affinity of metal ions for N-7 in the guanine residue (pK=2.11) is about 10 times higher than for N-7 in the adenine residue (pK= -0.2) (Sigel et al., 1994). In



Scheme 3. Possible reaction mechanism of the oligomerization of compound **6a**. The hydrolysis of the phosphoimidazolide groups could be assisted by an intramolecular attack - directly or via water molecules - of the protonated endocyclic nitrogen atoms. This would result in an acceleration of the condensation reaction. It should be noted that the endocyclic N-atom chosen for protonation in this mechanism is arbitrary.

biological pyrimidine nucleotides Mg^{2+} or Mn^{2+} ions are coordinated only with the phosphate groups (Pezzano and Podo, 1980). The reason is that in solution, the pyrimidine nucleotides exist predominantly in the *anti*-conformation, in which the N-3 is pointed away from the phosphate group. The metal ion cannot coordinate with the phosphate group and simultaneously with N-3 without a change of conformation of the nucleotide to the less favored *syn*-conformation (Massoud and Sigel, 1988). These conformational restrictions for pyrimidine nucleotides are not relevant for the analogues **1-6**. Therefore, it is supposed that Mn^{2+} and

Mg^{2+} may coordinate with the endocyclic N-atoms of the heterocyclic ring, directly or via a water molecule bridge, and simultaneously with the phosphate group of the analogues. It is concluded that this macrochelate formation is related to the number of endocyclic N-atoms that are available for interaction, while the basicities of these atoms are of minor importance. According to this hypothesis, the extent of macrochelate formation will increase in the order: **1a<5a<6a<3a,4a**, while cyclization decreases in the same order.

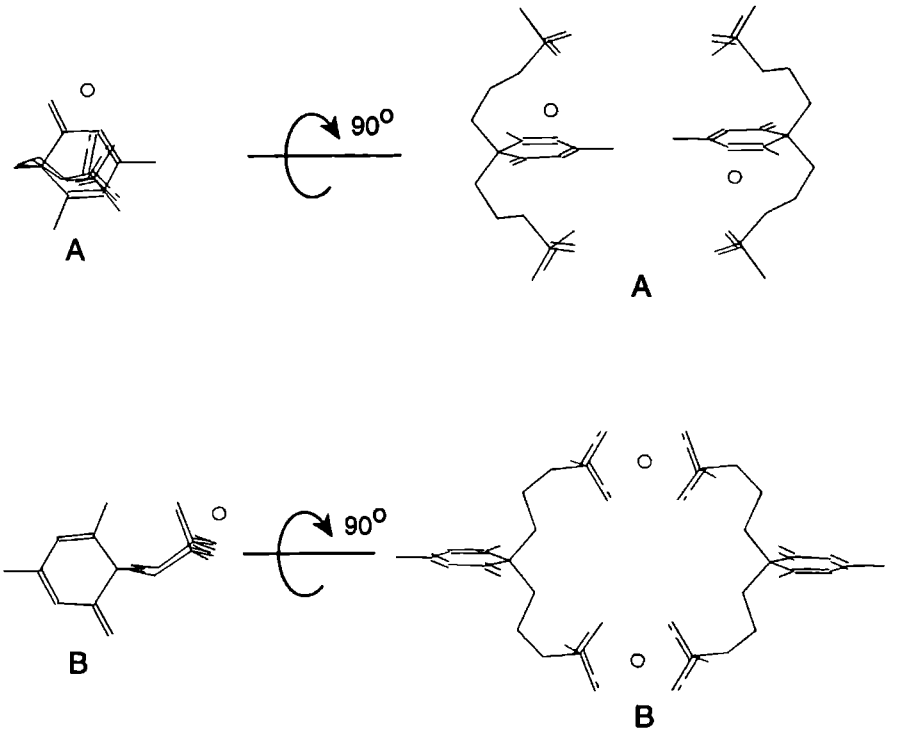


Figure 5. Schematic view of possible conformations of analogues **1-6** A) Conformation in which the phosphate-coordinated metal ions are also complexed with the heterocyclic ring Cyclization of two or more monomers is prohibited by steric hindrance of the heterocyclic rings B) Conformation in which the metal ions are coordinated only with the phosphate groups In this conformation cyclization is permitted and oligomerization is inhibited

Conclusions

The polymerization behaviors of the analogues **1a-6a** are dependent on the nature of the exocyclic groups as well as the pK-values of the heterocyclic rings. Keto-enol tautomerism in

the pyrimidine bases of analogues **2** (pK=7.9) and **3** (pK=6.1) seems responsible for reaction of the phosphoimidazolide groups with the exocyclic oxygen atoms of the heterocyclic ring. Consequently, oligomerization of analogue **2a** yielded cyclic compound **26** and reaction of analogue **3a** resulted in the formation of branched oligomers. In comparison, the reactions with analogue **6a** (pK=6.9) showed a rather clean oligomerization pattern. After protonation of the ring, this analogue probably exists only in the keto-form. Furthermore, the limiting factor for the production of high molecular weight oligomers seems to be the formation of cyclic oligomeric products. It is proposed that production of cyclic oligomers is related to the degree of complexation of phosphate-coordinated metal ions to the ring. If metal ions cannot coordinate with endocyclic N-atoms, as in the case of compound **1**, the formation of cyclic products will be substantial. If coordination of the metal ion with the endocyclic N-atom and simultaneously with the phosphate group is favored, the degree of cyclization will be limited by the extent of macrochelate formation.

Prebiotic Implications

We have shown above that for non-enzymatic polymerization of the nucleotide analogues studied, both the nature of the ring substituents and the ionization constants of the heterocyclic ring are important. At pH values at which enolization is favored, reactions with exocyclic oxygens lead to cyclization or formation of branched oligomers, rather than the formation of linear polymers which are necessary for template-directed replication. In comparison, the ionization constants of the natural nucleotides are smaller than 4.5 or higher than 9.4 (Sober, 1973). Under prebiotic conditions (pH 7-8), the heterocyclic rings of these nucleotides are consequently relatively resistant to modification. Any candidates as possible precursors of RNA molecules should therefore display similar properties.

Acknowledgement.
We thank B. Barbier for a gift of RPC-5.

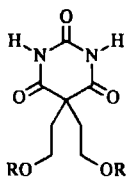
Polymerization Studies of Acyclic Nucleotide Analogues Related to Barbituric Acid in Mixtures of Organic Solvents and Water. Preparative Synthesis of Two Complementary Oligomers.

Abstract

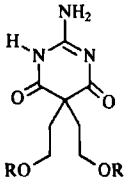
Oligomerizations of analogues **1a-6a** were studied in mixtures of organic solvents and water at 37°C. The yield of pyrophosphate-linked oligomers of **1** and **3-6** was higher than in similar reactions in water. These syntheses provide a simple method for producing high molecular weight pyrophosphate-linked oligomers for use in binding studies or in template-directed oligomerization studies. Oligomers of two potentially complementary analogues **1** and **4** were prepared and studied by UV-spectroscopy. Interactions between these oligomers appear to be weak in aqueous solution.

Introduction

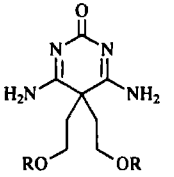
Achiral nucleotide analogues related to barbituric acid have been suggested as possible candidates for the role of precursors of the first RNA molecules (Figure 1) (Schwartz, 1993). To study the validity of these analogues it was decided to attempt to carry out nonenzymatic template-directed oligomerization reactions utilizing analogues **1a-6a**. For this purpose appropriate templates needed to be synthesized. Previously, pyrophosphate-linked oligomers of nucleotide analogues were synthesized by an efficient ring-opening polymerization of eight-member cyclic (internal pyrophosphate) monomers (Tohidi and Orgel, 1990; Visscher and Schwartz, 1990b). These monomers were prepared by cyclization of bisphosphoimidazolides of the various nucleotide analogues. The analogues shown in Figure 1, however, would



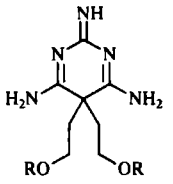
1 ; R=PO_3^{2-}
1a , R=PO_2 Im



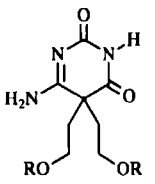
2 ; R=PO_3^{2}
2a , R=PO_2 Im



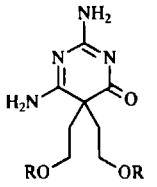
3 ; R=PO_3^{2}
3a ; R=PO_2 Im



4 ; R=PO_3^{2}
4a ; R=PO_2 Im



5 ; R=PO_3^{2-}
5a , R=PO_2 Im



6 , R=PO_3^{2-}
6a , R=PO_2 Im

PO_2 Im =

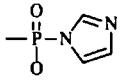


Figure 1.

produce ten-membered cyclic pyrophosphates, which are less likely to undergo ring opening oligomerization. Furthermore, a study of analogue **1a** in aqueous solution produced only a trace amount of an internal pyrophosphate-linked cyclic monomer (van Vliet et al., 1994b). This may be explained by the observation that formation of ten-membered cyclic rings are thermodynamically and entropically less favorable than formation of eight-membered rings (Illuminati and Mandolini, 1981). Methods have been developed to synthesize dimers with an internucleosidic pyrophosphate bond in organic solution using appropriate protecting and activating groups (Sekine et al., 1985; van der Woerd et al., 1987). However, these methods are not convenient for the synthesis of high molecular weight oligomers of **1-6**.

Because oligomers of **1-4** possess no stereoisomers and the internal cyclization of the monomers is very limited, we decided to study the oligomerization of the analogues **1a-6a** in mixtures of organic solvents and water, with divalent metal ions as promoters. Under optimized conditions, oligomers of two potentially complementary analogues **1** and **4** were synthesized preparatively and the interaction between these oligomers was measured by UV-spectroscopy.

Experimental

Proton nuclear magnetic resonance spectra were recorded with a Varian EM 390 or Bruker 400 MHz spectrometer, ^{31}P-NMR spectra (proton decoupled) with a Bruker 400 MHz spectrometer. Ultraviolet spectra were recorded with a Beckman DU-40 spectrophotometer using cells of 1.0 or 10 mm pathlength. Q-Sepharose (Fast Flow) was purchased from Pharmacia. Pyrophosphatase (type II from *Crotalus adamanteus* venom), and alkaline phosphatase (type III from *Escherichia coli*) were from Sigma. HPLC analysis of oligomers was carried out on an RPC-5 column in 0.02 M NaOH with a linear gradient of $NaClO_4$ (0.03 M over 45 min for reaction products of **1**, **3** and **5** and 0.018 M over 45 min for reaction products of **4** and **6**) at 1.0 ml/min. The eluent was monitored by a variable wavelength uv detector. Triethylammonium bicarbonate (TEAB) buffer was made by bubbling carbon dioxide gas through a stirred mixture of triethylamine (TEA, 825 ml) and water (1175 ml) at 0°C until the pH of the clear solution was 7.5. The compounds **1-6** and **1a-6a** were synthesized as described earlier (van Vliet et al., 1994a, 1994b; Chapters 2 and 5).

Oligomerization reactions in mixtures of organic solvents and water.

All reactions were performed in Eppendorf tubes in a total volume of 10 μl. Firstly, a solution of $MgCl_2$ (2 or 4 μl, 1.0 M) or $MnCl_2$ (4 μl, 1.0 M) was added to each tube and the solutions

were evaporated to dryness. To the residues an organic solvent was added, followed by a freshly prepared cold solution of activated monomer in an imidazole-HCl buffer (pH 6.5) at 0°C. If necessary the solutions were diluted with water to a total volume of 10 μl. Finally, the solutions were mixed, centrifuged and incubated for 5 days at 37°C. The reactions were quenched by addition of KEDTA (8 μl, 1.0 M, pH 9.0), diluted with water to a volume of 100 μl and stored at -25°C. For HPLC-analysis aliquots with a volume of 10 μl were taken from the mixtures. Surviving imidazolides were hydrolyzed by incubation in a sodium acetate (NaAc) buffer (pH 4.0, 0.1 M, 100 μl) for 1 h at 50°C. The reaction conditions are shown in Tables 1-5.

Oligomerization of compound **1a** *to produce oligomer* **1**.

Compound **1a** (140 μmol) was suspended in a mixture of pyridine (370 μl) and water (6 μl) and incubated for 4 days at 50°C. The reaction mixture was evaporated and coevaporated twice with water, after which the reaction was analyzed by HPLC on an RPC-5 column. The residue was treated with a solution of Tris-HCl (6.0 ml, 0.04 M, pH 8.0) containing 0.02 M $MgCl_2$ and 20 units alkaline phosphatase to remove terminal phosphate groups. After stirring for 4 h at 37°C the reaction was quenched by addition of KEDTA (240 μl, 1.0 M, pH 9.0) and the mixture was diluted with a TEAB solution (200 ml, 0.1 M, pH 8). Separation of the oligomers was over a Q-Sepharose column with a linear gradient of TEAB (0.1-2.0 M, pH 8). Fractions containing oligomers with chain lengths n ($16 \leq n \leq 18$; and $n>18$) were isolated. Yield (n>18) as determined by UV was 27.1 μmol (19 %). ^{1}H-NMR (D_2O, external TMS) δ ppm: 2.208-2.238 (t, CH_2 (without phosphate), J=5.9 Hz), 2.311 (s (br), 4H, 2×CH_2), 3.516-3.542 (t, OCH_2 (without phosphate), J=5.9 Hz), 3.831 (s (br), 4H, 2×OCH_2). ^{31}P-NMR (D_2O, external trimethylphosphate) δ ppm: -11.383 (s (br)).

Oligomerization of compound **4a** *to produce oligomer* **4**.

A cold and freshly prepared solution of compound **4a** (175 μmol) in imidazole-HCl buffer (300 μl, 1.0 M, pH 6.5) was added to a suspension of $MgCl_2.6H_2O$ (122 mg, 0.6 mmol) in pyridine (1.2 ml). A clear solution was formed and the reaction mixture was incubated for 5 days at 37°C. The reaction was quenched by addition of an KEDTA solution (600 μl, 1.0 M, pH 9.0), followed by evaporation of the reaction mixture and coevaporation twice with water. The reaction was analyzed by HPLC on an RPC-5 column. The residue was treated with a solution of Tris-HCl (9.0 ml, 0.04 M, pH 8.0) containing 0.02 M $MgCl_2$ and 20 units alkaline phosphatase to remove terminal phosphate groups. After stirring for 4 h at 37°C the reaction was quenched with KEDTA (360 μl, 1.0 M, pH 9.0) and the mixture was diluted with a TEAB solution (200 ml, 0.02 M, pH 8). Separation of the oligomers was over a Q-Sepharose

column with a linear gradient of TEAB (0.1-1.0 M, pH 8). Fractions containing oligomers with chain lengths n ($16 \leq n \leq 18$; and $n>18$) were isolated. Yield (n>18) as determined by UV was 43 μmol (25 %). ^{1}H-NMR (D_2O, external TMS) δ ppm: 2.329-2.363 (t, CH_2 (without phosphate), J=5.8 Hz), 2.452-2.480 (t, 4H, 2xCH_2, J=5.6 Hz), 3.483-3.519 (t, OCH_2 (without phosphate), J=5.8 Hz), 3.813 (s (br), 4H, 2xOCH_2). ^{31}P-NMR (D_2O, external trimethylphosphate) δ ppm: -11.740 (s (br)).

Identification of isolated oligomers.

The length of the isolated oligomers was determined by HPLC analysis on an RPC-5 column. Isolated oligomers were degraded by $ZrCl_4$ and by pyrophosphatase to establish the length of the oligomers. For example, a pentamer was hydrolyzed with $ZrCl_4$ producing the tetramer, trimer, dimer and monomer in increasing proportions with time. Similar results were achieved by digestion with pyrophosphatase.

Enzyme digestions and chemical hydrolysis.

Pyrophosphatase digestions were performed on isolated oligomers (0.05 ODU) in a Tris-HCl buffer (0.1 M, 100 μl, pH 7.2) containing $MgCl_2$ (0.04 M) with 0.2 units enzyme. Incubation was for 5 h at 37°C. Alkaline phosphatase treatment was performed on samples in a Tris-HCl buffer (0.04 M, 100 μl, pH 8.0) containing $MgCl_2$ (0.02 M) with 0.2 units enzyme for 4 h at 37°C. After incubation KEDTA (4 μl, 1.0 M, pH 9.0) was added and the mixtures were analyzed by HPLC.

Chemical hydrolysis of the pyrophosphate linkages was performed by treatment with $ZrCl_4$. To samples of isolated oligomers (75 μl, 0.05 ODU) a solution of NaAc (13 μl, 3.0 M, pH 5.0) and $ZrCl_4$ (3 μl, 1.0 M) were added and the mixtures were incubated for various times at 50°C. The reactions were quenched by addition of KEDTA (9 μl, 1.0 M, pH 9.0) and neutralized with NaOH (6.7 μl, 10 M). After filtration the mixtures were analyzed by HPLC.

UV-measurements.

Absorbance spectra were measured for solutions of oligomer **1**, oligomer **4** and of an equimolar mixture of the two oligomers. The chain length of the oligomers was 16-18. Three series of experiments were performed in which the concentrations of each of the oligomers were 0.1, 0.67 and 1.0 mM (theoretical monomer equivalent). The solutions contained $MgCl_2$ (0.1 M), NaCl (1.0 M) and Tris-HCl (0.2 M, pH 7.0). Each series of experiments was performed at 4, 20 and 40°C. To ensure complete complex formation the reaction mixtures were stored overnight at 4°C, followed by 10 minutes at the appropriate temperature before the absorbance spectra were recorded.

Hydrolysis of oligomer **4** *with $ZrCl_4$ for determination of hyperchromicity.*
All reactions were performed in 2.0 ml polyethylene tubes (Eppendorf). To each tube a solution of oligomer **4** (0.2 μmol, chain length 16-18) in sodium acetate buffer (2.0 ml, 0.04 M, pH 4.5) was added, followed by $ZrCl_4$ (8 μl, 1M). The tubes were mixed and incubated at 50°C for 0, 1, 2.5 and 5 h. UV absorbance spectra were measured, after which the reactions were quenched with KEDTA (1.0 M, 24 μl), neutralized with NaOH (10 M, 15 μl) and filtered. The degree of degradation was determined by HPLC on an RPC-5 column.

Results and Discussion

Oligomerization studies in mixtures of organic solvents and water.
Reaction of the analogues **1a**, **3a-6a** produced an extensive series of pyrophosphate-linked oligomerization products in mixtures of organic solvents and water. The reaction conditions and the results are presented in Tables 1-5. The yields of high molecular weight oligomers were much higher than in aqueous solution (Chapter 5). Experiments were conducted in 20, 50 and 80% DMF to study the effect of the different proportions of DMF on the oligomerization reaction. Tables 1-5 show that the extent of oligomerization increases as the proportion of DMF in the reaction mixture is raised. The lower polarity of the reaction mixture may be responsible for the increased yield of oligomerization products. In protic solvents - like water - phosphate groups are strongly solvated, decreasing the nucleophilicity of these anions. Mixtures of aprotic solvents - like DMSO or DMF - and water have a diminished capacity to stabilize the charge of phosphate groups and as a consequence the nucleophilicity of the phosphate groups is enhanced in those mixtures. In other words, the phosphate groups are at a higher energy level due to a decreased electrostatic solvent stabilization (Carey and Sundberg, 1990).

Reactions were also performed in 80% DMSO and 80% pyridine. The degree to which organic solvents increased the yield of products was dependent on the nature of the ring of the analogue. In most cases oligomerizations in mixtures containing DMF gave better results than in mixtures containing DMSO or pyridine. The variation in efficiency of the reactions of the different analogues in the various mixtures of organic solvents with water could also partially be due to variations in electrostatic solvent stabilization of the phosphate groups. A second mode of interaction in these cases could be structural effects, which may cause reactants or transition states to be particularly strongly solvated by hydrogen bonds. The relative energies of the ground state and the transition state could be changed and cause rate variations from solvent to solvent (Carey and Sundberg, 1990).

Table 1. Product distributions in the oligomerization of analogue **1a** in mixtures of organic solvents and water.[a]

Metal	solvent	Monomer (%)	Relative yield of oligomers of length *n* (%)				
			$n \geq 2$	$n \geq 5$	$n \geq 10$	$n \geq 15$	$n \geq 20$
0.4 M Mg	20% DMF	14	86	25	2.4		
0.2 M Mg	50% DMF	4	96	53	33	25	19
0.4 M Mg	50% DMF	3	97	47	23	15	11
0.2 M Mg	80% DMF	2	98	53	47	44	26
0.4 M Mg	80% DMF	1	99	42	29	26	25
0.4 M Mg	80% Pyr	7	93	48	26	15	8
0.4 M Mg	80% DMSO	3	97	36	18	13	11
0.4 M Mn	80% DMF	1	99	62	55	53	50

Table 2. Product distributions in the oligomerization of analogue **3a** in mixtures of organic solvents and water.[a]

Metal	solvent	Monomer (%)	Relative yield of oligomers of length *n* (%)				
			$n \geq 2$	$n \geq 5$	$n \geq 10$	$n \geq 15$	$n \geq 20$
0.4 M Mg	20% DMF	21	79	67	43	28	18
0.2 M Mg	50% DMF	25	75	70	60	54	50
0.4 M Mg	50% DMF	20	80	75	62	55	50
0.2 M Mg	80% DMF	25	75	67	57	50	46
0.4 M Mg	80% DMF	35	65	60	47	41	33
0.4 M Mg	80% Pyr	15	85	78	62	49	40
0.4 M Mg	80% DMSO	11	89	82	67	58	52
0.4 M Mn	80% DMF	22	78	74	65	58	52

[a]Conditions: 0.05 M monomer; 0.2M/0.4 M imidazole-HCl buffer (pH 6.5); 4 days at 37 °C.

Table 3. Product distributions in the oligomerization of analogue **4a** in mixtures of organic solvents and water.[a]

Metal	solvent	Monomer (%)	Relative yield of oligomers of length *n* (%)				
			$n \geq 2$	$n \geq 5$	$n \geq 10$	$n \geq 15$	$n \geq 20$
0.4 M Mg	20% DMF	16	84	40	13	5	2
0.2 M Mg	50% DMF	7	93	67	39	23	13
0.4 M Mg	50% DMF	5	95	67	51	38	28
0.2 M Mg	80% DMF	2	98	85	74	64	55
0.4 M Mg	80% DMF	2	98	82	75	69	63
0.4 M Mg	80% Pyr	5	95	80	57	38	25
0.4 M Mg	80% DMSO	4	96	54	43	37	32
0.4 M Mn	80% DMF	3	97	80	66	54	45

Table 4. Product distributions in the oligomerization of analogue **5a** in mixtures of organic solvents and water.[a]

Metal	solvent	Monomer (%)	Relative yield of oligomers of length *n* (%)				
			$n > 2$	$n \geq 5$	$n \geq 10$	$n > 15$	$n \geq 20$
0.4 M Mg	20% DMF	19	81	27	4.1		
0.2 M Mg	50% DMF	13	87	56	42	33	24
0.4 M Mg	50% DMF	10	90	48	37	30	23
0.2 M Mg	80% DMF	26	74	58	52	46	41
0.4 M Mg	80% DMF	20	80	47	39	34	29
0.4 M Mg	80% Pyr	13	87	66	44	28	18
0.4 M Mg	80% DMSO	14	86	36	22	17	14
0.4 M Mn	80% DMF	9	91	76	70	64	58

[a]Conditions: 0.05 M monomer; 0.2M/0.4 M imidazole-HCl buffer (pH 6.5); 4 days at 37 °C.

Table 5. Product distributions in the oligomerization of analogue **6a** in mixtures of organic solvents and water.[a]

Metal	solvent	Monomer (%)	Relative yield of oligomers of length *n* (%)				
			$n \geq 2$	$n > 5$	$n \geq 10$	$n \geq 15$	$n \geq 20$
0.4 M Mg	20% DMF	12	88	47	17	7	2.7
0.2 M Mg	50% DMF	11	89	73	53	38	27
0.4 M Mg	50% DMF	11	89	76	55	44	35
0.2 M Mg	80% DMF		[b]				
0.4 M Mg	80% DMF	10	90	79	68	60	51
0.4 M Mg	80% Pyr	11	89	75	47	28	17
0.4 M Mg	80% DMSO	12	88	71	59	50	45
0.4 M Mn	80% DMF	5	95	88	79	72	67

[a]Conditions: 0.05 M monomer; 0.2M/0.4 M imidazole-HCl buffer (pH 6.5); 4 days at 37 °C.
[b]The yield of oligomers was something lower as in the reaction with 0.4 M $MgCl_2$, but could not be determined exactly.

The effect of the divalent cation was also investigated by performing experiments with 0.2 M Mg(II), 0.4 M Mg(II) and 0.4 M Mn(II). From Tables 1-5 it is observed that in the oligomerizations of compound **1a** and **5a** the yield of longer oligomers is lower at the higher concentration of Mg(II). In the case of the compounds **4a** and **6a** the opposite effect was observed. If these results are compared with the formation of cyclic pyrophosphate-linked short oligomers a distinct relation is observed, as is illustrated in Figure 2. If the cyclization of oligomers is relatively high, as in the reaction of the compounds **1a** and **5a**, the formation of high molecular weight oligomers is less efficient when the Mg(II) concentration is raised. If the cyclization is low, as in the oligomerization of the compounds **4a** and **6a**, the yield of longer oligomers increases with increasing concentration of Mg(II). These results suggest that the limiting factor in the oligomerization reaction is the production of cyclic oligomers, which is in agreement with the results of the oligomerization reactions in aqueous solution (Chapter 5). This assumption is also supported by the results of the reactions performed in the presence of Mn(II). In Figure 3 the production of longer oligomers and the yield of cyclic oligomers in the presence of Mg(II) or Mn(II) is given. The oligomerization of compounds **1a**, **5a** and **6a** produced more high molecular weight oligomers with Mn(II), while more cyclic products

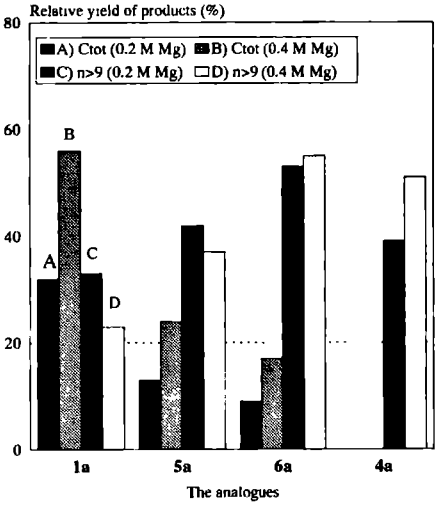


Figure 2. Relation between the product distributions in the oligomerizations of the analogues **1a**, **4a-6a** and the concentration of Mg(II). Conditions: 0.05 M activated monomer, 0.2/0.4 M $MgCl_2$ and 0.4 M imidazole-HCl buffer (pH 6.5) in DMF/water (1:1, v/v) for 5 days at 37°C.
Ctot = total extent of cyclic oligomers; n>9 = oligomers of lengths n>9.

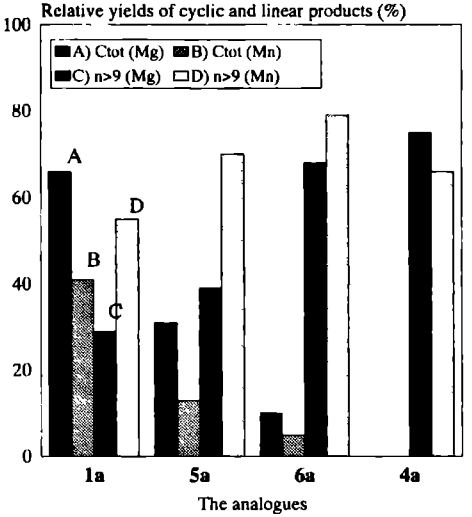
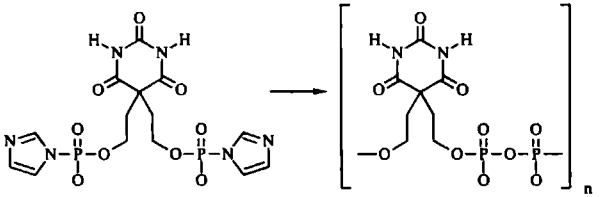
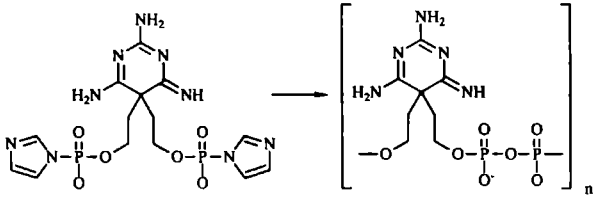


Figure 3. Relation between the product distributions in the oligomerizations of the analogues **1a**, **4a-6a** and the concentration of divalent metal ions Mg(II) and Mn(II). Conditions: 0.05 M activated monomer, 0.4 M MCl_2 and 0.4 M imidazole-HCl buffer (pH 6.5) in DMF/water (1:1, v/v) for 5 days at 37°C.
Ctot = total extent of cyclic oligomers; n>9 = oligomers of lengths n>9.

were formed with Mg(II). If no cyclic products are formed, as in the oligomerization of compound **4a**, the yield of longer oligomers is even higher with Mg(II). If the relation between the cyclization and the nature of the heterocyclic ring is compared, it is noticed that the cyclization is inversely correlated with the number of nonprotonated endocyclic N-atoms (Figure 2 and Figure 3), which is in agreement with the behavior of the analogues in aqueous solution. The production of cyclic oligomers was supposed to be dependent on the degree of complexation of the phosphate-coordinated metal ions with the endocyclic N-atoms of the ring (Chapter 5). This suggestion is supported by the observation that reaction with Mn(II) produced less cyclic pyrophosphate-linked oligomers than with Mg(II), since the affinity of Mn(II) for the endocyclic N-atoms is higher than that of Mg(II) (Pezzano and Podo, 1980; Fraústo da Silva and Williams, 1991; Sigel et al., 1994).



1a oligomer **1a**



4a oligomer **4a**

Scheme 1.

It is interesting that the differences in yield between reactions in mixtures of organic solvents and water in the presence of Mg(II) or Mn(II) are much smaller than between those reactions in aqueous solution. In those reactions in which little cyclization was observed in 80% DMF, the presence of Mg(II) or Mn(II) produced comparable yields of oligomers as is illustrated in Figure 3. These results suggest that under such conditions the kinetic difference between reactions in the presence of Mn(II) and Mg(II) is very small.

Under most conditions oligomerizations of compound **3a** yielded the highest production of longer oligomers. HPLC chromatograms showed a complex pattern of peaks (not

shown) and because of the resemblance with chromatograms in aqueous solution (Chapter 5), they are also ascribed to branched oligomers. An exception was the reaction in 80% pyridine which showed a clear pattern of peaks on HPLC (not shown). A possible explanation is the lower polarity of 80% pyridine compared with 80% DMSO or 80% DMF. As a consequence, the pK-values of the heterocyclic ring would increase in pyridine, resulting in a lower extent of deprotonation of the heterocyclic ring (Corfu and Sigel, 1991; van Esch et al., 1994) and production of fewer branched oligomers.

Analogue **2a** gave almost no oligomerization products (not shown). Reaction of the phosphoimidazolide groups with the ring as described in Chapter 5 could be responsible for these results.

In summary, the reactions in mixtures of organic solvents and water may provide a simple method for the preparation of oligomers **1** and **3-6** for use in binding studies or in template-directed oligomerization studies.

Preparative synthesis of oligomers **1** *and* **4**.

Pyridine-water mixtures were chosen as solvent, because pyridine is easily removed by evapo-

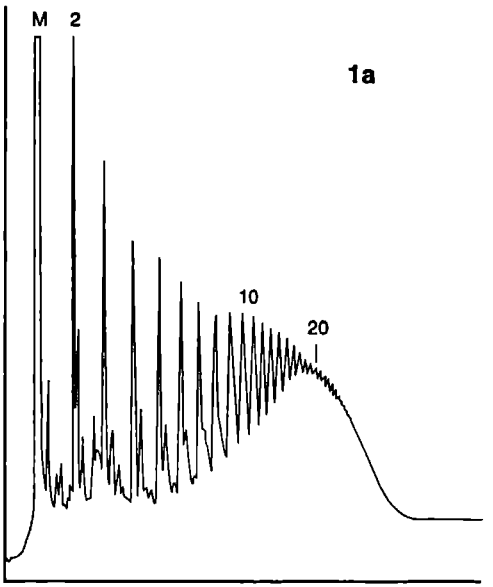


Figure 4. HPLC-chromatogram on RPC-5 of the preparative synthesis of oligomers of **1**.

ration. Oligomerization of compound **1a** yielded pyrophosphate-linked oligomers of **1** (Scheme 1) with chain lengths of 10 up to 40 (48%, Figure 4). Compound **4a** was oligomerized (yielding oligomer **4**) in a mixture of pyridine and imidazole-HCl buffer (pH 6.5) containing $MgCl_2$ as promoter. Oligomers with chain lengths of 10 and longer were formed in 63% yield. Both sets of oligomers were treated with alkaline phosphatase to remove terminal phosphate groups and fractionated on a Q-Sepharose column. The ^{31}P-NMR spectra of both sets of oligomers showed only one signal at -11.4 (**1**) and -11.7 (**4**) ppm, respectively, from which it is concluded that only pyrophosphate bonds were formed. The ^{1}H-NMR spectra of the alkaline phosphatase-treated fractions showed the expected upfield shifts of the terminal CH_2-groups due to dephosphorylation. Oligomers with a length of 18 and longer were used for template reactions described in Chapter 7.

UV measurements with oligomers **1** *and* **4**.

Oligomers (length 16-18) of **1** and **4**, which are theoretically capable of forming complementary pairs (Schwartz, 1993), were studied by UV spectroscopy. Measurements of

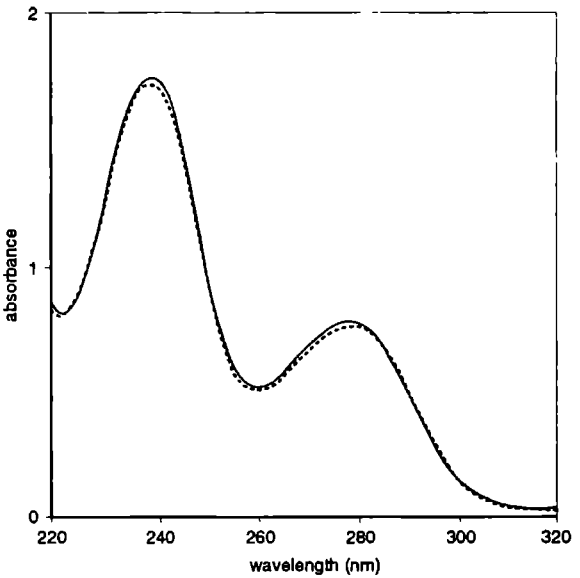


Figure 5. UV spectra at 4°C. Shown is the sum (dashed curve) of the two individual spectra of oligomer **1** and oligomer **4**, and the spectrum of an equimolar mixture of both oligomers (solid curve). Conditions: 0.67 mM of each oligomer (monomer equivalent), 0.1 M $MgCl_2$, 1.0 M NaCl and 0.2 M Tris-HCl (pH 7.0).

mixtures showed no detectable hypochromicity (Figure 5). Because hypochromicity can largely be ascribed to stacking interactions, intramolecular as well as intermolecular (Ts'o, 1974), we also checked for hyperchromicity as a result of the hydrolysis of oligomer **4** with Zr(IV) and found no such effect (Visscher and Schwartz, 1992). We therefore predict that complementary interactions between these analogues will only be observable in organic solvents, where hydrogen-bonding plays a more important role than stacking forces.

Acknowledgements.
We thank B. Barbier for a gift of RPC-5 and A.E.M. Swolfs for recording the NMR spectra.

Hydrogen-Bonding in the Template-Directed Oligomerization of a Nucleotide Analogue

Abstract

The oligomerization of monomers **4a** and **6a** (5,5-disubstituted derivatives related to 2,4,6-triaminopyrimidine) in the presence of the complementary, pyrophosphate-linked oligomer **1** (an oligomer of a 5,5-disubstituted derivative of barbituric acid) and of poly(U) was studied. Although oligomerization of monomer **4a** is unaffected by the presence of oligomer **1** or poly(U) with water as solvent, catalysis of the oligomerization is observed in N,N-dimethyl-formamide (DMF)/water (1:1, v/v). The oligomerization of monomer **6a**, isosteric in part to monomer **4a**, however, was only slightly stimulated in the presence of oligomer **1** in DMF/water (1:1, v/v). The results demonstrate that template catalysis of oligomerization of nucleotide analogues can occur under conditions which favor hydrogen-bonding, even in the absence of base-stacking effects.

Based in part on: M.J. van Vliet, J. Visscher and A.W. Schwartz. *J. Mol. Evol.* in press

Introduction

The well-known template-directed oligomerization (Inoue and Orgel, 1982) of mononucleotides depends upon the formation of a hydrogen-bonded, helical complex between a polynucleotide template and complementary monomers and oligomers. Under certain conditions, such as in the polycytidylic acid directed oligomerization of guanosine 5'-phosphoro(2-methyl)imidazolide, a double-stranded, Watson-Crick paired duplex is formed (Miles and Frazier, 1982). Other examples of catalysis which probably depend upon a combination of Watson-Crick and Hoogsteen base pairs have also been described (Huang and Ts'o, 1966; Inoue and Orgel, 1982).

The stabilities of nucleic acid duplexes in water depend to a major extent upon stacking interactions between neighboring bases. In organic solvents, where hydrogen bonds are many times stronger than in aqueous solution, a number of examples of self-organization and molecular recognition which depend solely on hydrogen bonds are known (Etter, 1990; Chang et al., 1991; Branda et al., 1994; Marsh et al., 1994; Mathias et al., 1994; Nowick et al., 1994). However, there are relatively few reports of such complex formation being utilized to catalyze a chemical reaction. Catalysis of a bimolecular reaction has been reported (Kelly et al., 1989), and especially interesting are examples of self-replicating dimer formation (Tjivikua et al., 1990; Terfort and von Kiedrowski, 1992).

We have reported the synthesis and oligomerization in aqueous solution as well as in organic solvents, of several new nucleotide analogues, which can be regarded as derivatives of barbituric acid (van Vliet et al., 1994a, 1994b). Oligomers **1** and **4** were prepared by oligomerization of the phosphoimidazolides in pyridine-water, or DMF-water mixtures at pH 6.5 (Figure 1). After removal of terminal phosphates by treatment with alkaline phosphatase, the products were fractionated on Q-Sepharose to isolate a fraction of oligomers with chain lengths greater than 18. Oligomers were characterized by ^{1}H-NMR and ^{31}P-NMR spectroscopy and by enzymatic and Zr-catalyzed hydrolysis as previously described (van Vliet et al., 1994a; Chapter 6).

A spectroscopic study of oligomers **1** and **4**, which could in theory form a hydrogen-bonded complex, detected no decrease in extinction coefficient on mixing in aqueous solution, nor was hyperchromicity observed upon hydrolysis of oligomer **4** (van Vliet et al., 1994a). The weakness of the stacking interactions between the pyrimidine ring systems suggested by these results, made it unlikely that template-directed oligomerization of monomer **4a** by oligomer **1** would be successful in aqueous solution, a conclusion which we have verified in this paper. Because decreasing the polarity of the solvent might sufficiently increase the role of hydrogen-bonding (Petersen and Led, 1981) so as to favor a template-directed oligomeri-

zation, we conducted experiments in less polar solvents.

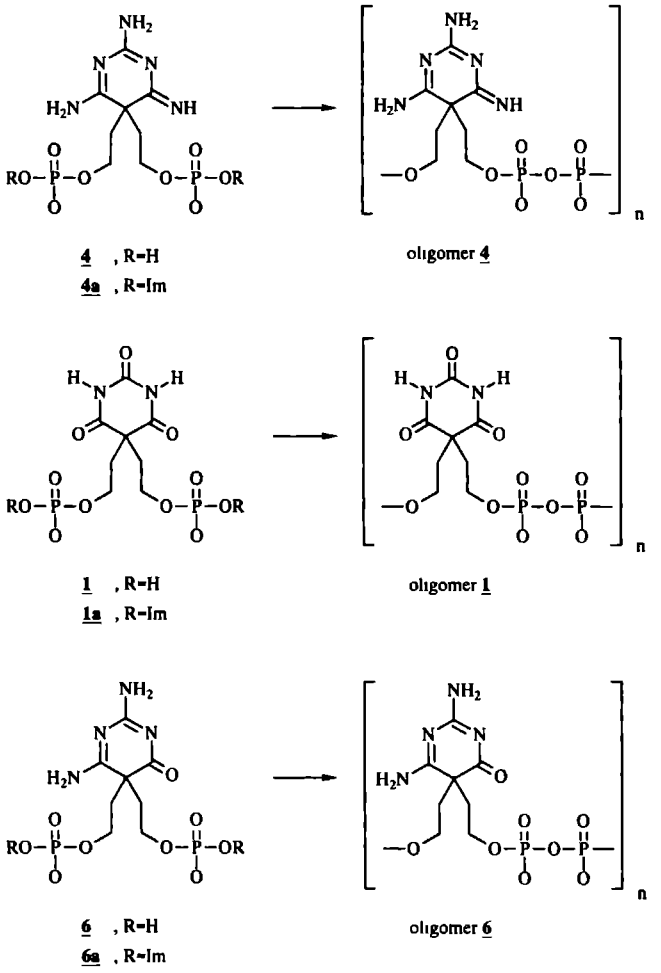


Figure 1. The structures of monomers and oligomers studied

Experimental

Material and methods

Ribonuclease (type I-A from bovine pancreas) and polyuridylic acid (poly(U)) were purchased

from Sigma. HPLC was performed on an RPC-5 column in 0.02 M NaOH with a linear gradient of $NaClO_4$ (0-0.012 M over 30 min) at a flow rate of 1.0 ml/min. The eluent was monitored at a wavelength of 270 or 280 nm depending on the nature of the component. The synthesis of the monomers **1a**, **4a** and **6a** has been described previously (van Vliet et al., 1994a, 1994b; Chapters 2 and 5). Oligomer **1** with a chain length n>18 was synthesized and isolated as described earlier (van Vliet et al., 1994a; Chapter 6).

Oligomerization reactions in aqueous solution.

All reactions were performed in Eppendorf tubes in a total volume of 10 μl. First solutions of oligomer **1** (37 μl, 0.027 M), $MgCl_2$ (4.0 μl, 1.0 M) and NaCl (1.0 μl, 1.0 M) were added to each tube. The mixtures were concentrated to dryness and the residues were dissolved in imidazole-HCl buffer (4.0 μl, 1.0 M pH 6.5). Then a freshly prepared, cold solution of monomer **4a** or **6a** (6 μl, 0.17 M) was added to each tube at 0°C and the contents were mixed. After centrifugation the reaction mixtures were incubated at 1°C for several weeks. The reactions were quenched by addition of 2 equivalents KEDTA per divalent metal ion, diluted with water to a total volume of 100 μl, and stored at a temperature of -25°C. Before HPLC-analysis aliquots with a theoretical monomer content of 0.05 μmol were taken from the mixtures. Surviving imidazolides were hydrolyzed by incubation in sodium acetate (NaAc) buffer (pH 4.0, 0.1 M, 100 μl) for 1 h at 50°C.

Reactions without oligomer **1** were incubated under identical conditions. Experiments were also conducted with a higher metal ion concentration (0.6 M $MgCl_2$ and 1.0 M NaCl). Reactions were also studied in the presence of the polynucleotide poly(U) using essentially the same procedure. Before HPLC-analysis poly(U) was destroyed by ribonuclease digestion. Ribonuclease digestions were performed on samples (0.05 μmol monomer equivalent) of the quenched reaction mixtures in Tris-HCl buffer (0.05 M, 100 μl, pH 7.6) containing 10 units of enzyme and incubated for 4 h at 37°C.

Oligomerization reactions in water-DMF mixtures.

Each reaction was performed in an Eppendorf tube. The following procedure was used for most experiments. Solutions of oligomer **1**, if required, and $MgCl_2$ were added to each tube, and evaporated to dryness. To the residues was added DMF followed by a freshly prepared solution of activated monomer in imidazole-HCl buffer (pH 6.5) at 0°C. The reactions were mixed thoroughly, centrifuged and incubated at 1 or 20°C for various times. The reactions were quenched and prepared for HPLC analysis as described above.

All reactions contained 0.4 M $MgCl_2$, 0.4 M imidazole-HCl buffer (pH 6.5), 0.1 μmol monomer **4a** or **6a** and oligomer **1** (0, 1, 2 or 4 monomer equivalents) in 1:1 (v:v) water/DMF. The

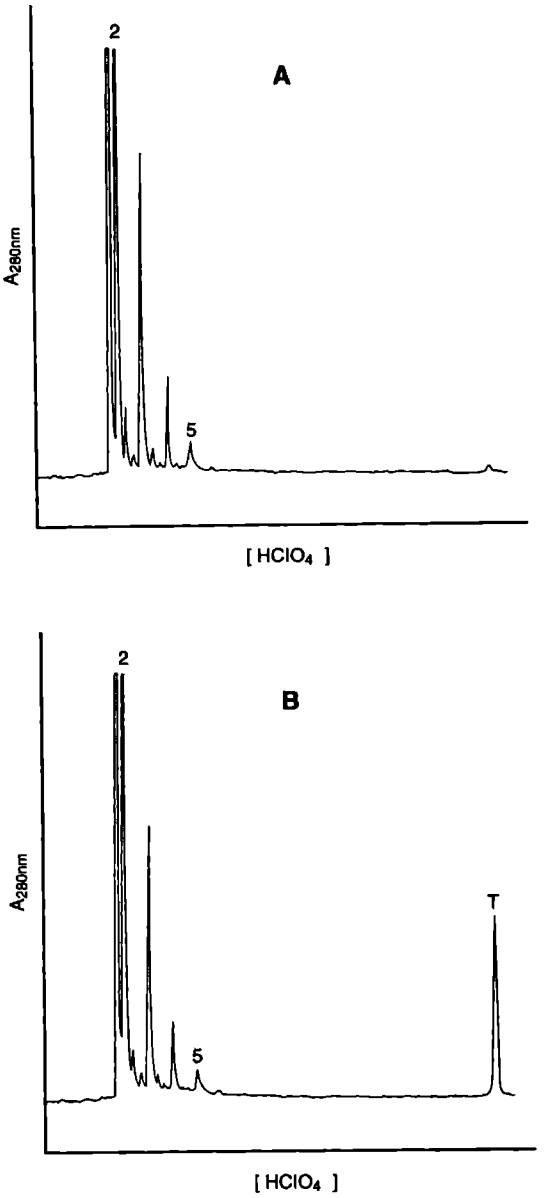


Figure 2. HPLC-chromatograms of oligomerization products of monomer **4a** in aqueous solution. A) Monomer **4a** alone, B) monomer **4a** in the presence of one equivalent of oligomer **1**. Conditions: 0.1 M monomer, 0.6 M $MgCl_2$, 1.0 M NaCl and 0.4 M imidazole-HCl (pH 6.5), 8 weeks at 1°C. The peak labeled T is due to elution of the template (oligomer **1**). Dimer and pentamer are marked in the chromatogram.

concentration of the monomer was reduced by increasing the volume of the reaction mixtures. Thus the volume of the reaction mixtures was 10 μl with a monomer concentration of 0.01 M, 40 μl with 0.0025 M monomer, and 100 μl with 0.001 M monomer. Reactions were also carried out in 20% and 80% DMF. The reactions in 80% DMF contained 0.2 M imidazole-HCl buffer (pH 6.5). Control experiments were conducted in which oligomer **1** was replaced by monomeric **1**, as the unphosphorylated bis-hydroxy compound (1,2 and 4 equivalents). The whole series of reactions was also performed in the presence of poly(U) (1, 2 and 4 monomer equivalents) using essentially the same procedure. Before HPLC-analysis poly(U) was destroyed by ribonuclease digestion.
Reactions were also conducted using DMSO instead of DMF.

Results and Discussion

Oligomerization of monomer **4a** *in the presence of oligomer* **1**.
First we studied the oligomerization of monomer **4a** in aqueous solution. HPLC-chromatograms of the products in the absence and presence of oligomer **1** are presented in Figure 2. As was expected from the results of a spectroscopic study of oligomers **1** and **4** (van Vliet et al., 1994a; Chapter 6) the extent of the oligomerization was unaffected by the presence of oligomer **1**, indicating that a stable complex was not formed.

To study the effect of a decreased polarity of the solvent on the template-directed oligomerization, we performed experiments in 50% DMF. The results summarized in Table 1 show that the oligomerization of monomer **4a** in 50% DMF is indeed stimulated by the addition of 1, 2 and 4 equivalents of oligomer **1**. Figure 3 compares the HPLC analyses of the products obtained with 0 and 2 equivalents of the oligomer. The yields of the longest products were most strongly increased. After 5 weeks reaction at 1°C, for example, the yield of oligomers with lengths of 5 or more increased four-fold in the presence of two equivalents of oligomer **1**, although the total yield of all oligomers was almost the same (44 and 45%). Inspection of the chromatograms suggests that it is primarily oligomers (dimers and longer) that condense to form longer products in the presence of the template (Figure 4). Essentially the same results were obtained when the oligomerization was conducted at 20°C for two days.

Oligomer **4** is expected to be capable of forming a complex with two equivalents of oligomer **1**, as in Figure 5 (A), although self-association of both analogues is also possible (B). The largest increase in the yield of the longest oligomers occurs after addition of two equivalents of oligomer **1** (Table 1). The continued - although more moderate - increase in extent of oligomerization observed as the concentration of oligomer **1** is increased from two to four

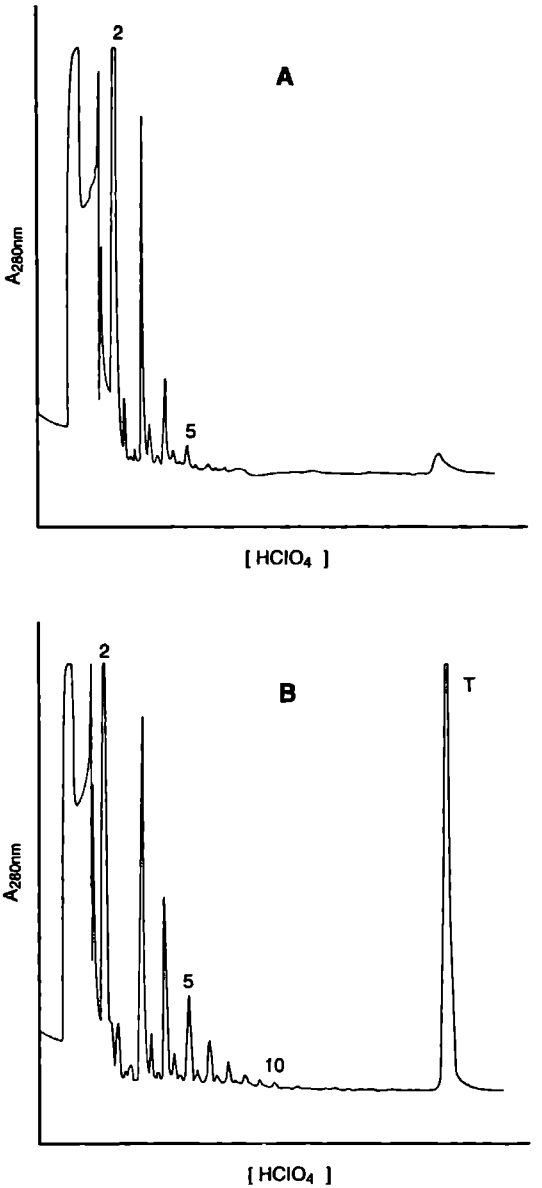


Figure 3. HPLC-chromatograms of oligomerization products of monomer **4a** in DMF/H_2O (1:1, v/v). A) Monomer **4a** alone, B) monomer **4a** in the presence of two equivalents of oligomer **1**. Conditions: 0.0025 M monomer, 0.4 M $MgCl_2$ and 0.4 M imidazole-HCl (pH 6.5), 5 weeks at 1°C. The minor peaks present between the labeled oligomers are due to the presence of a few percent of a by-product produced during imidazolization of the monomer. The peak labeled T is due to elution of the template (oligomer **1**). Oligomers with length 2, 5 and 10 are marked in the chromatogram.

Table 1. Product distributions in the oligomerization of monomer **4a** in 50% DMF.[a]

Oligomer **1** (M)	Poly(U) (M)	Monomer (%)	Relative yield of oligomers of length *n* (%)				
			$n \geq 2$	$n > 3$	$n \geq 4$	$n \geq 5$	$n \geq 10$
-	-	56	44	12	4.9	2 0	
0 0025	-	55	45	15	8	3 8	0.3
0 005	-	55	45	23	13	8	0 7
0 01	-	53	47	26	16	10	0 8
-	0 0025	54	46	15	7	2.6	
-	0 005	56	44	14	7	2.4	
-	0.01	53	47	16	7	2 8	

[a] Conditions 0 0025 M monomer **4a**, 0 4 M $MgCl_2$ and 0.4 M imidazole-HCl buffer (pH 6 5) in DMF/water (1:1, v/v) for 5 weeks at 1 °C.

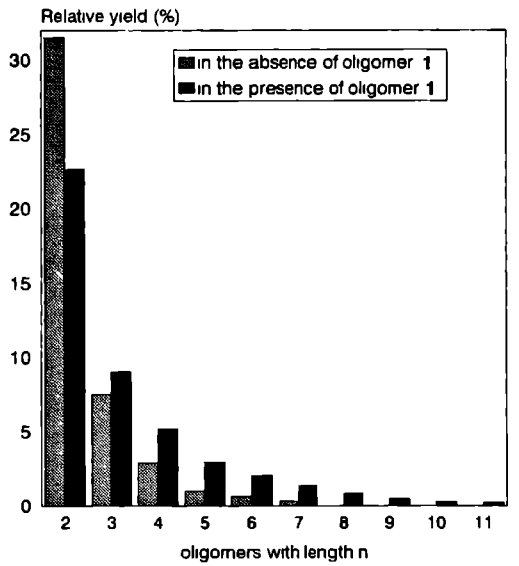


Figure 4. Product distribution in the oligomerization of monomer **4a** in 50% DMF. The increase of oligomers with length $n \geq 3$ is approximately as great as the decrease of oligomers with length n=2 This result suggests that oligomers with a length of 2 or longer are condensed to form longer products in the presence of the template Conditions: 0.0025 M monomer **4a**, 0.4 M $MgCl_2$ and 0 4 M imidazole buffer-HCl (pH 6 5) in DMF/water (1.1, v/v) for 5 weeks at 1 °C

equivalents probably results from competition between self-association of the template and complex formation between oligomers **1** and **4**. In a series of control experiments, we found that adding 1 to 4 equivalents of monomeric **1** (as the unphosphorylated bis-hydroxy compound) had no effect on oligomerization of monomer **4a**. This observation supports the conclusion that the catalysis is due to a template effect exerted by oligomer **1** (i.e., the condensation being favored along the chain) rather than through the formation of two-dimensional hydrogen-bonded sheets similar to those observed for barbituric acid-triaminopyrimidine (Lehn et al., 1990) or cyanuric acid-melamine (Seto and Whitesides, 1990) complexes.

The overall effect of the oligomer **1** template on the oligomerization decreases if the concentration of the monomer **4a** is raised from 0.0025 to 0.05 M. After 5 weeks and at a monomer concentration of 0.05 M only a small increase in the extent of oligomerization was visible, while after 8 weeks even a negative effect was observed (data not shown). A possible cause could be the efficient oligomerization of monomer **4a** at higher concentrations. At a monomer concentration of 0.05 M oligomerization of monomer **4a** in the absence of a template produces oligomers with lengths up to 30. The template used, with chain lengths of 18 to 30 monomer **1** units (Chapter 6), is probably too short for stimulating the template-directed elongation of these oligomers.

Oligomerization experiments with monomer **4a** were also conducted in 20% and 80% DMF. Under neither condition was a positive template effect observed in the presence of oligomer **1** (data not shown). In the template-directed oligomerization of monomer **4a** on oligomer **1** in 50% DMF we have suggested a competition between the complex formation of oligomer **4** with oligomer **1** and the self-complementary structure of oligomer **1**. In 80% DMF the polarity of the solvent may be too low to dissociate the self-complementary structure of oligomer **1** and favor the formation of a complex with newly synthesized short oligomer **4**. In contrast, the polarity of 20% DMF may not be low enough to permit the formation of a base-paired complex between small oligomers of **4** and the template. An additional contributory factor in this case is, no doubt, the lower extent of oligomerization in 20% DMF (Chapter 6).

To support the view that the stimulation of the oligomerization of monomer **4a** in the presence of a template in 50% DMF is promoted by the decreased polaritiy of the solvent and is not specific to DMF, we also conducted some experiments in DMSO. The polarities of both solvents are similar[1] and, as expected, comparable results were obtained (data not shown).

[1]Although the dielectric constant of DMSO (ϵ=47) is substantially higher than that of DMF (ϵ=37), another empirical measurement of solvent polarity, based on shifts in the absorption spectrum of a reference dye, showed comparable values for DMSO (45.0) and DMF (43 8) (Reichardt, 1979).

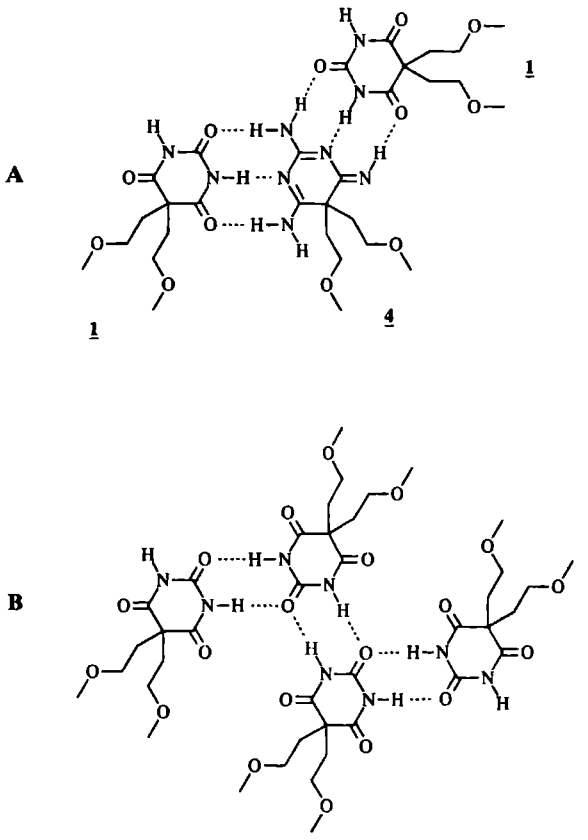


Figure 5. A) Possible structure of a hydrogen-bonded complex of oligomers **1** and **4**. B) Possible self-complementary structure of oligomer **1**.

Oligomerization of monomer **4a** *in the presence of poly(U).*

Because of the resemblance of uracil to the barbituric acid-like rings of oligomer **1**, we conducted oligomerization experiments with monomer **4a** in the presence of poly(U). In 50% DMF little stimulation of the reaction was observed at a monomer concentration of 0.0025 M (Table 1), but at a concentration of 0.01 M an effect was observed (Table 2). After 5 weeks at 1°C the total conversion of monomers to oligomers increased from 69 to 76% in the presence of poly(U), and the yield of oligomers with lengths of 10 and more increased by about two-fold. However, in contrast with the oligomerization experiments in which oligomer **1** acted as a template, addition of 2 or 4 equivalents of poly(U) produced no further stimulation of the reaction of monomer **4a**. This latter observation is in accord with the known properties of

poly(U), for which self-complementary structures are negligible.

The rather moderate effect of the poly(U) template contrasts with earlier results obtained in the oligomerization of acyclic nucleotide analogues related to glycerol (Visscher and Schwartz, 1989). However, oligomer **4** is structurally further removed from poly(U) than were the earlier analogues and constraints in the backbone of both oligomers may disfavor a conformation in which a hydrogen-bonded complex is possible. Moreover, in the presence of poly(U), which seems to act as a weaker template than oligomer **1**, an increase in the yield of oligomers of all lengths is obtained, rather than a preferential effect on longer oligomers. This suggests that condensation of monomer **4a** units can occur on regions of poly(U) that are free of self-associations, whereas for chain-extension by means of the condensation of oligomer **4** units on oligomer **1**, at least dimers are necessary to compete with the self-complementary structure of the template (see Figure 4).

Table 2. Product distributions in the oligomerization of monomer **4a** in 50% DMF.[a]

Oligomer **1** (M)	Poly(U) (M)	Monomer (%)	Relative yield of oligomers of length n (%)				
			$n \geq 2$	$n \geq 3$	$n \geq 5$	$n \geq 10$	$n \geq 15$
-	-	31	69	41	16	1.8	0.14
0.01	-	30	70	44	19	3.2	0.6
-	0.01	25	75	47	22	4.4	0.9
-	0.02	24	76	45	22	4.2	0.9
-	0.04	24	76	46	23	4.7	1.0

[a] Conditions: 0.01 M monomer **4a**, 0.4 M $MgCl_2$ and 0.4 M imidazole-HCl buffer (pH 6.5) in DMF/water (1:1, v/v) for 5 weeks at 1 °C.

Oligomerization of monomer **6a**.

We also investigated the oligomerization of monomer **6a** in the presence of oligomer **1** as well as in the presence of poly(U); both in aqueous solution and in 50% DMF. The heterocyclic ring of monomer **6a** is isosteric in part to monomer **4a** and can form three H-bonds with the heterocyclic ring of oligomer **1** or poly(U). As expected, the oligomerization of 0.1 M monomer **6a** showed no stimulation in the presence of either template in aqueous solution (data not shown). The results of the oligomerization in 50% DMF are presented in Table 3. After 5 weeks at 1°C and at a monomer concentration of 0.0025 M only a small increase in the formation of oligomer **6** was observed in the presence of oligomer **1**. Poly(U) did not affect

the reaction of monomer **6a** (0.0025 M or 0.01 M) at all. We suggest that these results are a consequence of the ionization constant of the heterocyclic ring of monomer **6**, which has a value of 6.9 (Chapter 5). At pH 6.5 a large fraction of the compounds will be protonated at one of the endocyclic N-atoms. If the endocyclic N-atom (N-3) between the two amino groups is protonated, a hydrogen-bonded complex between oligomer **1** and oligomer **6** or poly(U) will be disfavored, as is shown in Figure 6.

Table 3. Product distributions in the oligomerization of monomer **6a** in 50% DMF.[a]

Oligomer **1** (M)	Poly(U) (M)	Monomer (%)	Relative yield of oligomers of length *n* (%)			
			n≥2	*n*≥3	*n*≥4	*n*≥5
-	-	45	55	33	16	5.9
0.0025	-	42	58	33	16	7.3
0.005	-	39	61	37	19	9.3
-	0.0025	43	57	33	16	6.2
-	0.005	41	59	34	16	6.7

[a] Conditions: 0.0025 M monomer **6a**, 0.4 M $MgCl_2$ and 0.4 M imidazole-HCl buffer (pH 6.5) in DMF/water (1:1, v/v) for 5 weeks at 1°C.

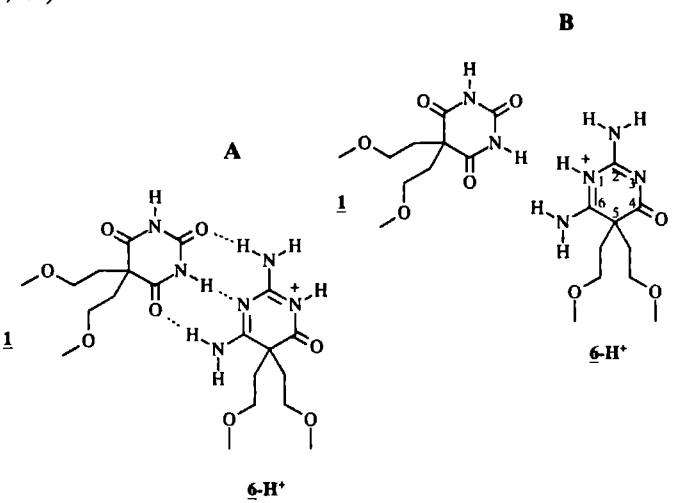


Figure 6. Possible tautomers of the ring of oligomer **6**-H$^+$ and the influence on the formation of an H-bonded complex. If N(3) is protonated, 3 H-bonds between the oligomer **6**-H$^+$ and **1** are possible (A); if N(1) is protonated this complex is impossible (B).

Conclusions and Discussion

In this work we studied the oligomerization of monomers **4a** and **6a** in the presence of the complementary oligomer **1**. Oligomerization of monomer **4a** in aqueous solution is unaffected by the presence of oligomer **1**, probably due to the lack of stacking interactions between the pyrimidine ring systems. Decreasing the polarity of the solvent by conducting oligomerizations in 50% DMF resulted in a demonstrable catalysis of the reaction by oligomer **1**. We conclude that stacking interactions between mononucleotide analogues will play a central role in determining the effectiveness of template-directed oligomerization in aqueous solution in the absence of a sophisticated catalyst, but that increased H-bonding can partially compensate for poor stacking. Several alternative possibilities for achieving a similar effect, which do not depend upon the use of an organic solvent, have been suggested in the literature (Constant et al., 1987; Kurihara et al., 1991; Nowick and Chen, 1992). At least two kinds of environments could have existed on the prebiotic Earth (Deamer and Pashley, 1989). Vesicles and micelles are known to effectively concentrate reactants into apolar internal compartments. In one case this effect has been demonstrated by the formation of hydrogen-bonded base pairs between derivatives of adenine and uracil (Nowick and Chen, 1992). An additional possibility is suggested by the observation that monolayers at an air-water interface display enhanced hydrogen bonding and nucleotides and nucleic acid bases have been shown to bind to diaminotriazine-functionalized monolayers on water (Bohanon et al., 1995).

From our study it is also suggested that ionization constants (pK-values) of the heterocyclic rings of nucleotide analogues are critical for molecular recognition, due to the fact that an H-bonded complex may be disfavored by protonation of one of the bases. As was earlier noted on the basis of different arguments (Chapter 5), it is therefore not surprising that the ionization constants of the heterocyclic rings of all the natural nucleotides in the genetic system have values smaller than 4.5 or larger than 9.4 (Sober, 1973). This may provide a clue for an (at least partial) answer to the question: "why did nature choose the natural heterocyclic bases as components of the genetic system".

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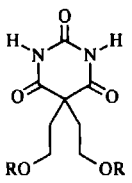
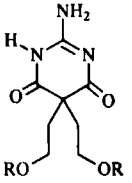
Summary and Conclusions

It is widely believed that life evolved from a set of self-replicating informational macromolecules. The template properties and catalytic ability of RNA has led to the idea that such a self-replicating system could have consisted of a set of RNA molecules. RNA is composed of mononucleotides, which consist of base, ribose and phosphate residues. However, no plausible prebiotic route has been found for the synthesis of nucleotide monomers and, especially, for the ribose residue. In addition, nonenzymatic transcription is inhibited by racemic mixtures of mononucleotides. For these reasons, it was suggested that RNA might have been preceded in evolution by a structurally simpler, achiral molecule. In our laboratory we have sought to test this hypothesis by performing experiments with nucleotide analogues in which ribose was



1 ; R=PO_3^{2-}
1a ; R=PO_2·Im



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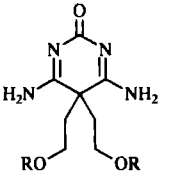
2a ; R=PO_2·Im



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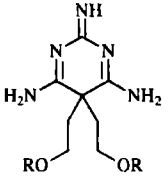

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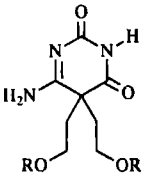

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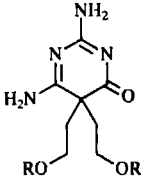


6 ; R=PO_3^{2-}
6a ; R=PO_2·Im

PO_2·Im =

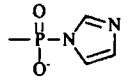


Figure 1.

replaced by a structure based on glycerol. In this thesis this study has been extended to the new set of nucleotide analogues shown in Figure 1. The analogues are 5,5-disubstituted pyrimidines related to barbituric acid. They are theoretically able to form base-paired complexes with each other, although self-complementary structures are also possible.

In Chapter 2 the synthesis of the six analogues is described. After bis-alkylation of the α,α-positions of diethylmalonate, malononitrile or ethylcyanoacetate, the corresponding protected pyrimidine analogues were formed by condensation with urea and guanidine, respectively. The protecting groups were removed followed by phosphorylation of the alcohol functions. From ^{1}H-NMR spectra and molecular modeling studies some information about the conformation of the analogues was obtained.

The conditions for oligomerization of compound **1** were studied as described in Chapter 3. The activated analogue **1a** undergoes extensive oligomerization in aqueous solution to produce pyrophosphate-linked chains. The oligomerization products were isolated and characterized by enzymatic and chemical hydrolysis. The influence of divalent metal ions, pH and temperature on the oligomerization reaction was studied. The extent of the oligomerization was found to depend strongly on the nature as well as on the concentration of the divalent metal ions. The best results were obtained in the presence of manganese ions at pH 6. Under conditions of high monomer concentration (0.1 M), more than 90% of the monomer is converted to oligomers with chain lengths exceeding 20. These results far exceed, both in chain length and yield, previous results obtained in the oligomerization of nucleotide analogues in the absence of a template. In contrast to a number of other bisphosphorylated nucleoside analogues which have been studied, the compound has little tendency to cyclize, which is one of the reasons for the effective oligomerization. The high activity of manganese ions in the reaction is probably due to a high affinity of these ions for complexes in which more than one oxygen or nitrogen atom is involved, as well as the relative lability of those complexes.

In Chapter 4 the influence of the clay montmorillonite on the oligomerization of analogue **1** is discussed. The bisphosphate **1** was oligomerized in aqueous solution by addition of a water soluble carbodiimide as condensating agent. Under dilute conditions (0.005 M monomer) and in the presence of Na^+-montmorillonite, pyrophosphate-linked oligomers up to the pentamer were produced, while in the absence of this clay mineral only a small amount of dimer was formed. The reaction was also stimulated by the presence of divalent cations, such as magnesium and manganese ions, and by imidazole. The oligomerization of the bisphosphoimidazolide **1a** was also catalyzed by the presence of Na^+-montmorillonite.

The determination of the ionization constants of all six nucleotide analogues as well as conditions for their oligomerization in aqueous solutions are described in Chapter 5. After activation of the monomers, pyrophosphate-linked chains were produced in high yield. An

exception was the behavior of analogue **2a**, in which an intramolecular reaction of the phosphoimidazolide groups with the exocyclic oxygen atoms of the heterocyclic ring resulted in the formation of a tricyclic compound. Oligomerization of compound **3a** yielded branched oligomers, due to an intermolecular reaction of the phosphoimidazolide groups with the ring. Keto-enol tautomerism in the pyrimidine bases seems responsible for these results. The limiting factor for the production of high molecular weight oligomers is probably the formation of cyclic oligomers. It is proposed that the production of these cyclic oligomers is inversely correlated to the degree of complexation of phosphate-coordinated metal ions to the ring.

The oligomerization of the analogues in mixtures of organic solvents and water is described in Chapter 6. The yield of pyrophosphate-linked oligomers of **1** and **3-6** was higher than in similar reactions in water. These syntheses provide a simple method for producing high molecular weight pyrophosphate-linked oligomers for use in binding studies or in template-directed oligomerization studies. Oligomers of two potentially complementary analogues **1** and **4** were prepared and studied by UV-spectroscopy. Interactions between these oligomers appear to be weak in aqueous solution.

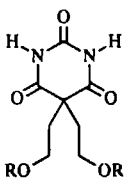
The template effect of the pyrophosphate-linked oligomer **1** as well as polyuridylic acid on oligomerization reactions of the monomers **4a** and **6a** is described in Chapter 7. Oligomerization of monomer **4a** in aqueous solution was unaffected by the presence of either the complementary oligomer **1** or polyuridylic acid, owing to the lack of stacking interactions between the pyrimidine ring systems. However, decreasing the polarity of the solvent by conducting oligomerizations in 50% dimethylformamide resulted in a demonstrable catalysis of the reaction by both oligomer **1** and polyuridylic acid. The oligomerization of monomer **6a**, isosteric in part to monomer **4a**, was only slightly stimulated in the presence of oligomer **1** in 50% dimethylformamide. This is probably due to protonation of an endocyclic N-atom, which disfavors a complex between oligomer **1** and oligomer **6**.

In conclusion, the results demonstrate that template-catalyzed oligomerization of nucleotide analogues can occur under conditions which favor hydrogen-bonding, even in the absence of base-stacking effects. This means that under those conditions nonenzymatic replication may be possible for systems consisting of pyrimidine residues only. Two different kinds of environments that could have existed on the prebiotic earth which would enhance H-bond formation are vesicles or micelles and air-water interfaces.

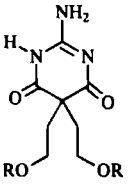
From our study it is also suggested that ionization constants (pK-values) of the heterocyclic rings of nucleotide analogues are critical for non-enzymatic polymerization as well as for molecular recognition. At pH-values at which enolization is favored, reactions with exocyclic oxygens lead to internal cyclization or formation of branched oligomers, rather than

the formation of linear polymers which are necessary for template-directed replication (Chapter 5). Furthermore, formation of an H-bonded complex may be disfavored by protonation of one of the bases (Chapter 7). It is therefore not surprising that the ionization constants of the heterocyclic rings of all the natural nucleotides in the genetic system have values smaller than 4.5 or larger than 9.4. Any candidates as possible precursors of RNA molecules should therefore display similar properties.

Algemeen wordt aangenomen dat het leven op aarde is voortgekomen uit een reeks macromoleculen die informatie bevatten en zichzelf konden reproduceren. Daar RNA zowel informatie kan overdragen als reacties kan katalyseren is het idee ontstaan dat zo'n zelf-reproducerend systeem uit een reeks RNA moleculen bestond. RNA bestaat uit nucleotiden die zijn opgebouwd uit base, ribose en fosfaat groepen. Er is echter nog geen aanvaardbare prebiotische route gevonden voor de synthese van deze nucleotiden. Vooral de synthese van ribose lijkt problematisch. Bovendien blijken racemische mengsels van mononucleotiden de niet-enzymatische replicatie van oligomeren te stoppen. Daarom is de hypothese ontstaan dat in de evolutie RNA is voorafgegaan door een systeem met een eenvoudiger struktuur bestaande uit achirale



<u>1</u> ; R=PO_3^{2-}
<u>1a</u> ; R=PO_2-Im



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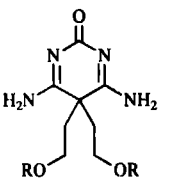
<u>2a</u> ; R=PO_2-Im



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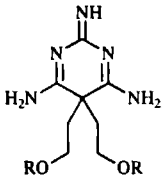
<u>3a</u> ; R=PO_2-Im



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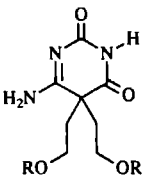

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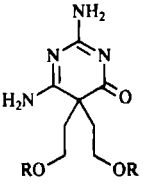
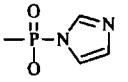


<u>6</u> , R=PO_3^{2-}
<u>6a</u> ; R=PO_2 Im

PO_2-Im =



Figuur 1.

moleculen. In dit laboratorium is deze hypothese onderzocht door middel van experimenten met nucleotide analogen waarin ribose is vervangen door een gemodificeerd glycerol molecule. Deze studie is in dit proefschrift voortgezet met een nieuwe reeks van nucleotide analogen die zijn weergeven in Figuur 1. De analogen zijn 5,5-digesubstitueerde pyrimidinen gerelateerd aan barbituurzuur. Theoretisch zijn ze instaat om waterstof-gebonden complexen met elkaar te vormen, maar ook zelf-complementaire strukturen behoren tot de mogelijkheden. Het doel van het huidige onderzoek was moleculaire replicatie na te bootsen door matrijs-gestuurde oligomerisatie reacties van enkele van deze nucleotide analogen uit te voeren.

In hoofdstuk 2 is de synthese van de zes verbindingen beschreven. Na bisalkylering van de α,α-posities van diethylmalonaat, malonitril of ethylcyanoacetaat werden door condensatie met urea of guanidine de betreffende beschermde pyrimidine analogen gevormd. De beschermende groepen werden afgesplitst waarna de vrijgekomen alcohol functies werden gefosforyleerd. Met behulp van ^{1}H-NMR spectra en 'molecular modeling' studies is enige informatie over de conformatie van deze verbindingen verkregen.

De condities voor oligomerisatie van verbinding **1** zijn bestudeerd als beschreven in hoofdstuk 3. Het geactiveerde analoog **1** condenseerde in waterige oplossing tot pyrofosfaat gebonden oligomeren. De oligomerisatieprodukten werden geïsoleerd en vervolgens gekarakteriseerd door middel van enzymatische en chemische hydrolyse. De invloed van tweewaardige metaalionen, pH en temperatuur op de oligomerisatie reactie werd bestudeerd. De mate van oligomerisatie was sterk afhankelijk van de aard alsmede van de concentratie van de tweewaardige metaalionen. De beste resultaten werden met mangaanionen verkregen bij pH 6.0. In een geconcentreerde oplossing (0,1 M monomeer) werd meer dan 90% van de monomeren omgezet in oligomeren met ketenlengten van meer dan 20 nucleotiden. Deze resultaten zijn veel beter, zowel in ketenlengte als in opbrengst, dan die van tot nu toe bekende oligomerisatie reacties van nucleotide analogen waarbij geen matrijs aanwezig was. In tegenstelling tot een aantal andere bestudeerde bisgefosforyleerde nucleotide analogen cycliseren deze verbindingen nauwelijks, wat een reden is voor de effectieve oligomerisatie. De grote activiteit van mangaanionen wordt waarschijnlijk veroorzaakt door de hoge affiniteit van dit metaalion voor complexen, waarbij stikstof en meerdere zuurstof atomen betrokken zijn, en de relatieve labiliteit van deze complexen.

In hoofdstuk 4 werd de invloed van het kleimineraal montmorilloniet op de oligomerisatie van verbinding **1** bestudeerd. Oligomerisatie van het bisfosfaat **1** werd uitgevoerd in waterige oplossing door toevoeging van een wateroplosbaar carbodiimide als condensatiemiddel. Bij een lage monomeer concentratie (0,005 M) en in de aanwezigheid van Na^+-montmorilloniet werden pyrofosfaat gebonden oligomeren met een maximale ketenlengte van vijf geproduceerd. Zonder kleimineraal werd alleen een kleine hoeveelheid dimeer gevormd. De

reactie werd gestimuleerd door tweewaardige kationen zoals magnesium- en mangaanionen en door imidazol. De oligomerisatie van de bisfosfoimidazolide **1a** werd ook gekatalyseerd in de aanwezigheid van Na^+-montmorilloniet.

De ionisatie constanten van de zes nucleotide analogen alsmede omstandigheden voor hun oligomerisatie in waterige oplossing zijn beschreven in hoofdstuk 5. Na activering van de monomeren vond een efficiënte reactie plaats waarbij lange oligomeren gevormd werden. Een uitzondering was de reactie van analoog **2a** waarbij door een intramoleculaire reactie van de fosfoimidazolide groepen met de exocyclische zuurstof atomen van de heterocyclische ring een tricyclische verbinding gevormd werd. Oligomerisatie van verbinding **3a** leverde vertakte ketens op als gevolg van een intermoleculaire reactie van de fosfoimidazolide groepen met de ring. Keto-enol tautomerisatie in de pyrimidine base lijkt verantwoordelijk voor deze resultaten. De beperkende factor voor de produktie van lange polymeerketens is de vorming van cyclische oligomeren. Wij veronderstellen dat de produktie van deze cyclische oligomeren omgekeerd evenredig is aan de mate van complexatie van de fosfaat-gecoördineerde metaalionen met de ring.

De oligomerisatie van de analogen in mengsels van organische oplosmiddelen en water is beschreven in hoofdstuk 6. De opbrengst aan oligomeren van **1** en **3-6** was hoger dan die van vergelijkbare reacties in water. Deze synthesen voorzien in een eenvoudige methode voor de produktie van lange pyrofosfaat gebonden oligomeren voor gebruik in bindingstudies of in matrijs-gestuurde oligomerisatie reacties. Oligomeren van twee potentiële complementaire analogen **1** en **4** werden preparatief gesynthetiseerd en bestudeerd met UV-spectroscopie. Interacties tussen deze oligomeren blijken in waterige oplossing zwak te zijn.

In hoofdstuk 7 is het matrijseffect van oligomeer **1** en van polyuridylzuur op de oligomerisatie reacties van de monomeren **4a** en **6a** bestudeerd. Oligomerisatie van monomeer **4a** in waterige oplossing werd niet beïnvloed door het complementaire oligomeer **1** of polyuridylzuur. De oorzaak ligt aan het gebrek aan stapelings (stacking) interacties tussen de pyrimidine ringsystemen. Als echter de polariteit van het oplosmiddel verlaagd werd, door oligomerisaties uit te voeren in 50% dimethylformamide, bleek de reactie door oligomeer **1** of polyuridylzuur duidelijk gekatalyseerd te worden. Het matrijseffect van oligomeer **1** op de reactie van monomeer **6a** in 50% dimethylformamide was zeer klein. Dit wordt waarschijnlijk veroorzaakt door protonering van een endocyclisch N-atoom, waardoor een complex tussen oligomeer **1** en **6** ongunstig is.

De resultaten laten zien dat zonder stapelingseffecten tussen de basen en onder omstandigheden waarin waterstofbindingen versterkt worden, er toch matrijs-gekatalyseerde oligomerisaties van nucleotide analogen kunnen plaatsvinden. Dit betekent dat onder die omstandigheden niet-enzymatische replicatie van systemen opgebouwd uit alleen pyrimidine

residuen tot de mogelijkheden behoord. Twee verschillende soorten omgevingen die H-bindingen versterken en die mogelijk op de prebiotische aarde aanwezig waren, zijn vesicels of micellen en lucht-water scheidingsvlakken.

Uit deze studie is ook geconcludeerd dat ionisatie constanten (pK-waarden) van de heterocyclische ringen van de verbindingen kritisch zijn voor niet-enzymatische polymerisatie alsmede voor moleculaire herkenning. Bij pH-waarden waarbij enolisatie bevorderd wordt, leiden reacties met exocyclische zuurstof atomen tot cyclisatie of produktie van vertakte oligomeren in plaats van tot de synthese van lineaire polymeren die nodig zijn voor matrijs-gestuurde replicatie. Bovendien kan door protonering van een van de basen de vorming van complexen gebaseerd op waterstofbindingen verhinderd worden. Het is daarom geen verrassing dat de ionisatie constanten van de heterocyclische ringen van alle natuurlijke nucleotiden in het genetische systeem kleiner zijn dan 4.5 of groter dan 9.4. Mogelijke voorlopers van RNA moleculen zullen daarom overeenkomstige eigenschappen moeten bezitten.

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Curriculum Vitae

Michiel van Vliet werd op 27 oktober 1963 geboren te Schiedam. Na het behalen van het VWO diploma in 1982 aan het St-Antonius college te Gouda begon hij met de studie scheikunde aan de Rijksuniversiteit Utrecht. Het propedeutisch examen werd in augustus 1983 behaald. Het doctoraal programma werd afgesloten op 30 mei 1988 en bestond uit het hoofdvak Bio-organische chemie (Prof. Vliegenthart en Prof. Kamerling) en de keuzevakken Bio-organische chemie en Biochemie. Vervolgens vervulde hij van september 1988 tot januari 1990 de militaire dienstplicht als groepscommandant bij de pantserinfanterie. Van april 1990 tot april 1994 was hij als assistent in opleiding verbonden aan de onderzoeksgroep Evolutiebiologie van de Katholieke Universiteit Nijmegen, waar onder leiding van Prof. Schwartz het in dit proefschrift beschreven onderzoek werd verricht. In deze periode werden verscheidene internationale congressen bezocht, waaronder 'the Gordon Conference on the Origins of Life' (New London, 1992; Rhode Island, 1994) en 'the 10th International Conference on the Origin of Life' (Barcelona, 1993). Op dit laatste congres werd ook een lezing gegeven. Van januari tot april 1995 was hij als onderzoeker werkzaam bij de werkgroep 'Synthese van biopolymeren' (Prof. Van Boom) van de Rijksuniversiteit Leiden.